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(54) Title: AN IMPROVED METHOD FOR HYBRIDIZING NUCLEIC ACIDS USING SINGLE-STRANDED NUCLEIC ACID BINDING PROTEIN			
(57) Abstract The present invention relates to an improved method for hybridizing polynucleotides with complementary nucleic acid sequences. Specifically, it relates to a method of increasing the specificity of a polynucleotide hybridization reaction in the presence of single-stranded nucleic acid binding protein.			

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AN IMPROVED METHOD FOR HYBRIDIZING
NUCLEIC ACIDS USING
SINGLE-STRANDED NUCLEIC ACID BINDING PROTEIN

Description

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Technical Field

The present invention relates to a process for hybridizing polynucleotides with complementary nucleic acid sequences. More particularly, the invention relates to the use of single-stranded nucleic acid binding protein in nucleic acid hybridization reaction admixtures to decrease nonspecific hybridization and thereby increase the specificity of a hybridization reaction.

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Background of the Invention

Nucleic acid hybridization technology is used as a fundamental process in many procedures of modern biotechnology and genetic engineering. Nucleic acid hybridization is a process in which a single-stranded nucleic acid pairs up with a complementary nucleotide sequence present on another nucleic acid forming hydrogen bonds between complementary nucleotide bases on the two paired strands of the classical double-stranded DNA helix. Because of the requirement that hybridized nucleic acid strands have complementary nucleotide base sequences, hybridization processes are used to locate, detect and/or isolate specific nucleotide base sequences present on target nucleic acids.

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Nucleic acid hybridization techniques have been applied to many procedures, including but not limited to Southern blot detection of specific nucleic acid sequences [Southern, J. Mol. Biol.,

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98:503-17 (1975)], library screening for cloning and manipulation of nucleic acid fragments into recombinant DNA cloning vectors [Maniatis et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (1982); and Ausubel et al, Current Protocols in Molecular Biology, John Wiley and Sons (1987)], and hybridization of polynucleotide primers in, for example, the polymerase chain reaction method to amplify specific nucleic acid sequences [U.S. Pat. No. 4,683,195 and No. 4,683,202; PCR Technology, Erlich, ed., Stockton Press (1989); and Polymerase Chain Technology, Erlich et al, eds., Cold Spring Harbor Laboratory Press (1989)], in dideoxy sequencing of nucleic acids, and in in vitro mutagenesis procedures.

Of particular importance in all nucleic acid hybridization procedures is the avoidance of nonspecific hybridization between strands of nucleic acid that do not have complementary nucleotide base sequences. See, for example, Beck et al, Nuc. Acid Res., 16:9051 (1988); and Haqqi et al, Nuc. Acid Res., 16:11844 (1988).

The process and specificity of hybridization between a single-stranded nucleic acid and another nucleic acid having a complementary nucleic acid sequence is known to be subject to a variety of conditions including temperature, the length and GC content of the nucleic acid sequences to be hybridized, and the presence of salts or additional reagents in the hybridization reaction mixture as may affect the kinetics of hybridization. Nonspecific hybridization arises when hybridization is carried out under conditions of low stringency in which noncomplementary (mismatched) nucleotide bases are paired in the resulting double-stranded DNA helix.

- Nucleic acid hybridization is typically used to produce primed nucleic acid synthesis templates. Primed templates are nucleic acids comprised of two nucleic acid strands of unequal length bound together to form a substrate for polynucleotide synthesis.

5 Primed templates are used in a wide variety of molecular biological techniques, including gene cloning, in vitro gene mutagenesis, nucleic acid amplification, nucleic acid detection, and the like.

10 Primed templates are typically produced by hybridizing (annealing) a primer to a target nucleotide sequence on the template that is complementary to the sequence of the primer. The fidelity of hybridization reactions of primer with

15 template is known to vary depending on a variety of factors, including temperature, complexity of the template, and the like. Generally, greater template length and higher hybridization temperatures each contribute to increased mismatching between primer

20 and template resulting in inappropriately and undesirably primed template.

Inappropriately primed template is the major cause of undesirable (background or secondary) primer extension reaction products in primer extension

25 reactions. This is particularly the case in the polymerase chain reaction (PCR) method for amplifying specific nucleic acid sequences. See, for example, Beck et al, Nuc. Acid Res., 16:9051 (1988); and Haqqi et al, Nuc. Acid Res., 16:11844 (1988).

30 In PCR, specific nucleic acid sequences are amplified using a chain reaction in which primer extension products are produced using primed nucleic acid templates. The product of each primer extension reaction specifically anneals with a primer and the

35 resulting primed template acts as a substrate for

further primer extension reactions. PCR is particularly useful in detecting nucleic acid sequences which are initially present in only very small amounts. However, the utility of PCR is often hampered by high levels of background primer extension reaction products due to primer/template mismatching. The procedure for conducting PCR has been extensively described. See United States Patents No. 4,683,195 and No. 4,683,202 both to Mullis et al.

Single-stranded nucleic acid binding proteins (SSB) have been characterized in some detail and include such members as the E. coli single-stranded binding protein (Eco SSB), T4 gene 32 protein (T4 gp32), T4 gene 44/62 protein, T7 SSB, coliphage N4 SSB, adenovirus DNA binding protein (Ad DBP or Ad SSB), and calf thymus unwinding protein (UP1). Chase et al, Ann. Rev. Biochem., 55:103-36 (1986); Coleman et al, CRC Critical Reviews in Biochemistry, 7(3):247 - 289 (1980); Lindberg et al, J. Biol. Chem., 264: 12700-08 (1989); and Nakashima et al, FEBS Lett. 43: 125 (1974).

SSB proteins have traditionally been viewed as functioning by minimizing secondary structure in ssDNA and thereby facilitating polymerase enzyme passage (processivity) along the DNA template. SSB proteins have been used in a variety of ways based on this property of SSB proteins.

Eco SSB is an SSB that increases the fidelity of DNA replication and stimulates E. coli DNA polymerases II and III but not polymerase I or T4 DNA polymerase. Chase et al, Ann. Rev. Biochem., 55:103-36 (1986). Eco SSB has been shown to relieve pausing by DNA polymerase III assemblies at regions of secondary structure [(LaDuca et al, Biochem.,

22:5177-87 (1983)] and in vitro studies of RecA-mediated reaction suggest that SSB affects ssDNA by removing secondary structures. Muniyappa et al, Proc. Natl. Acad. Sci. USA, 81:2257-61 (1984).

5 Zapoliski et al, in published PCT Patent Application No. W085/05685, describe the use of Eco SSB in combination with E. coli RecA protein and ATP to form a hybridization mixture having the capacity to stimulate the transfer of ssDNA to homologous
10 duplexes. The Zapoliski disclosure indicates that a cooperative binding between Eco SSB and the ssDNA increases the homologous pairing mediated by RecA.

 Christiansen et al, J. Mol. Biol., 115:441-54 (1977) describe the use of Eco SSB to catalyze
15 formation of a double-stranded DNA helix from complementary strands of bacteriophage lambda DNA. The Christiansen disclosure demonstrates that Eco SSB preferentially increases the rate of reassociation of
20 large complementary strands of about 50,000 nucleotide bases in length when compared to smaller complementary strands of about 200 nucleotide bases in length.

 T4 Gene 32 protein is an SSB coded for by gene 32 of bacteriophage T4. One of its functions is to
25 assist T4 DNA polymerase synthesis across regions of secondary structure in a single-stranded template. Huberman et al, J. Mol. Biol., 62:39-52 (1971). It has also been used in an in vitro mutagenesis reaction to promote uninterrupted synthesis from
30 template by addition of gene 32 protein to the template after a mutagenized primer is annealed but before polymerization. Muta-Gene M13 in vitro mutagenesis kit instructions, version #189 89-0096, p.34, Bio-Rad laboratories, Richmond, CA.

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- Kaspar et al, Nuc. Acids Res., 17:3616 (1989) describes the use of T4 gp32 in the primer annealing and the primer extension steps of a dsDNA sequencing procedure. The Kaspar disclosure states that adding
5 T4 gp32 allows Klenow enzyme to read through a region that previously caused termination, which suggests that the utility of T4 gp32 in a sequencing protocol was in reducing secondary structure to allow the polymerase to continue down the template.

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Brief Summary of the Invention

A composition for enhancing polymerase chain reaction specificity, which composition comprises, in aqueous solution, single-stranded binding protein in
15 an amount sufficient to enhance the production of primary polymerase chain reaction products and a non-ionic detergent in an amount sufficient to stabilize Taq polymerase against degradation during polymerase chain reaction amplification, said composition being
20 free of nuclease activity. Preferably, the single-stranded nucleic acid binding protein is present at a concentration of at least 0.1 milligrams/milliliter and the non-ionic detergent is present at a concentration of at least 0.1 volume percent.

25 A preferred composition for enhancing polymerase chain reaction specificity comprises, in aqueous solution, 0.5 milligrams/ milliliter single-stranded binding protein, 0.1 milligrams/milliliter carrier protein, 50 mM tris(hydroxymethyl)
30 aminomethane; 500 mM NaCl; 1 mM ethylenediaminetetraacetic acid; 1 mM 2-mercaptoethanol; 50 volume percent glycerol, and 1 volume percent polyoxyethylene lauryl ether. The composition is free of nuclease activity and has a pH
35 value in the range of 7 to 8.

Brief Description of the Drawings

In the drawings forming a portion of this disclosure:

Figure 1 illustrates the sensitivity of a PCR amplification and detection system for specific nucleic acids when conducted as described in Example 2. Primer extension products produced by PCR were analyzed by electrophoresis on 6% acrylamide gels, after individual reaction mixtures were produced containing the following amounts of lambda transgene mouse genomic DNA: 1 pg (lane 1), 10 pg (lane 2), 100 pg (lane 3), 1 ng (lane 4) 10 ng (lane 5), 50 ng (lane 6), 100 ng (lane 7) and 1 ug (lane 8). The arrow indicates the position of the band representing an amplified nucleic acid fragment that contains the specifically primed nucleic acid sequence. All other bands are the result of non-specific primer hybridization. Lane M contains molecular weight markers produced from a Hinf I restriction endonuclease digest of bacteriophage phi X174 DNA.

Panel A shows the results of a PCR amplification conducted in the absence of Eco SSB. Panel B shows the results of a PCR amplification under the same conditions as Panel A except that Eco SSB was included in the hybridization and primer extension reaction mixtures at 2.5 ug/ml.

Figure 2 illustrates the range of effectiveness of an SSB protein for improving a PCR-based specific nucleic acid detection system when conducted as described in Example 2. Primer extension products were produced in the presence of varying amounts of Eco SSB, and were analysed on 6% acrylamide gels as described in Figure 1. The amount of Eco SSB added per 100 ul of PCR mixture was: 10 ng (lane 1), 50 ng (lane 2), 100 ng (lane 3), 250 ng

(lane 4), 500 ng (lane 5), 1 ug (lane 6), 2 ug (lane 7), 5 ug (lane 8), 10 ug (lane 9), and zero ug (lane 10). The arrow indicates the position of the band representing an amplified nucleic acid fragment that contains the specifically primed nucleic acid sequence. All other bands are the result of non-specific primer hybridization. Lane 9 lacks primer extension products because of improper thermal cycling in that reaction well of the thermal cycler apparatus, or because 10 ug of Eco SSB may inhibit PCR.

Figure 3 illustrates an analysis of hybridization stringency in a PCR-based detection system when conducted as described in Example 2. Primer extension products produced by PCR using either a 42C (lanes 1-6) or 54C (lanes 7-12) annealing temperature were analyzed as described in Figure 1. PCR was conducted in the presence (+) or absence (-) or 2.5 ug Eco SSB per ml PCR mixture. Human genomic DNA was hybridized with primers pr 886 and pr 887 (lanes 1, 2, 7 and 8), and lambda transgene mouse genomic DNA (lanes 3-6 and lanes 9-12) was hybridized with an 18-mer primer pair, pr 737 and pr 738 (lanes 3, 4, 9 and 10), or with a 23-mer primer pair, pr 4266 and pr 19012 (lanes 5, 6, 11 and 12) in the PCR method. The upper arrow indicates the position of the 1800 bp specifically amplified nucleic acid fragment, and the lower arrow indicates the position of the 520 bp specifically amplified nucleic acid fragment. All other bands are the result of non-specific primer hybridizations. Lane M contains the same molecular weight markers as shown in Figure 1.

Figure 4 illustrates the use of two different primer pairs in a PCR-based detection method, as

described in Example 2, in which the primers have regions of non-homology with the specific target nucleic acid sequence. Primer extension products produced by PCR using the primer pair pr 1A and pr 1B (lanes 1 and 2) or using the primer pair pr 2A and pr 2B (lanes 3 and 4) were analyzed as described in Figure 1, and using the same markers in lane M. PCR was conducted in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of Eco SSB, and was directed at detecting the single copy DHFR gene present in human genomic DNA. The arrow indicates the position of the 250 bp band corresponding to a specifically amplified nucleic acid fragment, which is visible in lanes 2, 3 and 4, but not visible in lane 1.

Detailed Description of the Invention

A. Definitions

Nucleotide: a monomeric unit of DNA or RNA consisting of a sugar moiety (pentose), a phosphate, and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combination of base and sugar is a nucleoside. When the nucleoside contains a phosphate group bonded to the 3' or 5' position of the pentose it is referred to as a nucleotide. A sequence of operatively linked nucleotides is typically referred to herein as a "base sequence" or "nucleotide sequence", and is represented herein by a formula whose left to right orientation is in the conventional direction of 5'-terminus to 3'-terminus.

Duplex DNA: A double-stranded nucleic acid molecule comprising two strands of substantially complementary polynucleotide hybridized together by the formation of a hydrogen bond between each of the

complementary nucleotides present in a base pair of the duplex. Because the nucleotides that form a base pair can be either a ribonucleotide base or a deoxyribonucleotide base, the phrase "duplex DNA" refers to either a DNA-DNA duplex comprising two DNA strands (ds DNA), or an RNA-DNA duplex comprising one DNA and one RNA strand.

Base Pair (bp): a partnership of adenine (A) with thymine (T), or of cytosine (C) with guanine (G) in a double stranded DNA molecule. In RNA, uracil (U) is substituted for thymine.

Nucleic Acid: a polymer of nucleotides, either single or double stranded.

Gene: a nucleic acid whose nucleotide sequence codes for a RNA or polypeptide. A gene can be either RNA or DNA.

Complementary Bases: nucleotides that normally pair up when DNA or RNA adopts a double stranded configuration.

Complementary Nucleotide Sequence: a sequence of nucleotides in a single-stranded molecule of DNA or RNA that is sufficiently complementary to that on another single strand to specifically (non-randomly) hybridize to it with consequent hydrogen bonding.

Conserved: a nucleotide sequence is conserved with respect to a preselected (reference) sequence if it non-randomly hybridizes to an exact complement of the preselected sequence.

Hybridization: the pairing of complementary nucleotide sequences (strands of nucleic acid) to form a duplex, heteroduplex or complex containing more than two single-stranded nucleic acids by the establishment of hydrogen bonds between/among complementary base pairs. It is a specific, i.e. non-random, interaction between/among complementary

polynucleotides that can be competitively inhibited.

Hybridization product: The product formed when a polynucleotide hybridizes to a single or double-stranded nucleic acid. When a polynucleotide hybridizes to a double-stranded nucleic acid, the hybridization product formed is referred to as a triple helix or triple-stranded nucleic acid molecule. Moser et al, Science, 238:645-50 (1987).

Nucleotide Analog: a purine or pyrimidine nucleotide that differs structurally from a A, T, G, C, or U, but is sufficiently similar to substitute for the normal nucleotide in a nucleic acid molecule. Inosine (I) is a nucleotide that can hydrogen bond with any of the other nucleotides, A, T, G, C, or U. In addition, methylated bases are known that can participate in nucleic acid hybridization.

B. Methods

The present invention broadly contemplates an improvement to well known nucleic acid hybridization methods. The improvement comprises the use of SSB in the hybridization reaction admixtures to decrease the amount of nonspecific hybridization between complementary nucleic acids.

Because of the basic nature of the hybridization reaction in recombinant nucleic acid technologies, it is contemplated that the improvement can be practiced on other methods not specifically described herein that utilize hybridization as a part of the method. Therefore the following discussion is not intended to limit the present invention, but is provided to illustrate the application of principles that can be relied on to improve nucleic acid hybridization methods in general.

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1. Single-stranded Nucleic Acid Binding Protein

In all embodiments described herein there is a single-stranded nucleic acid binding protein (SSB) included in the hybridization reaction admixture.

"Single-stranded nucleic acid binding protein" as used herein refers to a class of proteins collectively referred to by the term SSB. Chase et al, Ann. Rev. Biochem., 55:103-36 (1986). (The art cited herein is hereby incorporated by reference.) SSB has the general property of preferential binding to single-stranded (ss) over double-stranded (ds) nucleic acids irrespective of the nucleotide sequence. The class includes such diverse members as Eco SSB, T4 gp32, T7 SSB, N4 SSB, Ad SSB, UP1, and the like. Chase et al, Ann. Rev. Biochem., 55:103-36 (1986); and Coleman et al, CRC Critical Reviews in Biochemistry, 7(3):247-289 (1980).

SSB has a strong binding affinity for ssRNA almost as well as ssDNA, and binds dsDNA substantially less well. For example, Eco SSB in general binds ssDNA ten times better than ssRNA, and 10^3 times better than dsDNA. Chase et al, Ann. Rev. Biochem.; 55:103-36 (1986).

A preferred SSB for use in the methods disclosed herein is Eco SSB or T4 gp32. More preferred and exemplary of the disclosed methods is Eco SSB, whose preparation and use is described herein.

SSB has other general properties described by Chase et al, Ann. Rev. Biochem., 55:103-36 (1986). These properties include the ability of an SSB to reduce the melting temperature of dsDNA, to increase processivity of an accessory DNA polymerase, to bind ssDNA stoichiometrically in an amount that depends on

the particular SSB protein, and to destabilize secondary structures in ssDNA.

The known SSBs all exhibit the characteristic of increasing the processivity of their accessory DNA polymerases. An SSB is deemed accessory to a DNA polymerase when both the SSB and the DNA polymerase are present in the same organism and characterized as having a capacity to stimulate a particular DNA polymerase. For example, Eco SSB is accessory to E. coli. DNA polymerase II and III because it stimulates that polymerase's activity, but does not stimulate T4 DNA polymerase.

Other SSBs useful in practicing the present invention are modified in amino acid residue sequence in some degree as to retain the general properties as described above. For example the protein produced by the mutant allele ssb-113 in E. coli. produces an SSB that binds to ssDNA as well as wild-type Eco SSB but exhibits a greater capacity to lower the melting temperature of dsDNA. Chase et al, J. Biol. Chem., 259:805-14 (1984). Therefore "modified" SSBs, derived by isolation of mutants or by manipulation of cloned SSB protein-encoding genes, are also contemplated for use in the presently disclosed methods.

An SSB as disclosed herein can be used alone or in combination with other SSBs in the disclosed methods.

An effective amount of one or more SSBs for use in the disclosed methods depends on the amount of nucleic acid present in the admixtures, as it is known that SSB binds to nucleic acids stoichiometrically. For example, Eco SSB binds ssDNA to a maximum of about one SSB binding site per 33 to 65 base nucleotides, depending upon the salt

concentration. Lohman et al, J. Biol. Chem.,
260:3594-603 (1985). Therefore, an effective amount
of SSB is typically in the range of 1 ng to 10 ug of
SSB protein per 100 ng of nucleic acid, preferably
5 about 0.1 ug to 5 ug, and more preferably about 0.25
ug to 2 ug of SSB protein per 100 ng nucleic acid.

To be effective in the presently contemplated
methods, SSB must be admixed with a nucleic acid in
the presence of an activating (effective) amount of
10 divalent cations. Preferred divalent cations for
activating SSB are Mg^{++} , Mn^{++} , Ca^{++} and Zn^{++} . More
preferably, Mg^{++} is used to activate SSB. An
activating amount of divalent cations is in the range
of 100 uM to 30 mM, and preferably 500 uM to 20 mM,
15 more preferably about 15 mM for hybridization
reactions. Christiansen et al, J. Mol. Biol.,
115:441-54 (1977). When SSB is used in a PCR-based
method it is preferred that the divalent cations be
present in the range of 1 to 5 mM, and more
20 preferably the divalent cation is Mg^{++} used at about
1.5 mM.

SSBs can be isolated from their source
organisms by standard biochemical methods involving
cell lysis and protein chromatography. Particularly
25 preferred are the methods described in Example 1
including ammonium sulfate and polyethylenimine
fractionation, affinity chromatography on ssDNA-
cellulose and phosphocellulose chromatography.
Alternatively, several of the SSBs are commercially
30 available.

Eco SSB can be obtained commercially from
Pharmacia, Inc. (Piscataway, NJ), or can be prepared
from a variety of E.coli strains, such as is
described by Chase et al, Nuc. Acid Res., 8:3215-27
35 (1980), or by Weiner et al, J. Biol. Chem., 250:1972-

80 (1975), or can purified by the the method disclosed in Example 1 using any of a variety of E. coli strains, including E. coli B or E. coli strain K12-H1-TRP containing the plasmid pTL119A-5.

5 T4 gp32 can be obtained commercially from Pharmacia, Bio-Rad (Richmond, CA) or Boeringer Mannheim Biochemicals (Indianapolis, IN), or can be prepared from a T4 infected bacterial culture such as is described by Jarvis et al, J. Biol. Chem.,
10 264:12709-16 (1989).

Because SSB is utilized in the methods of this invention in admixture with nucleic acids, it is preferred that SSB be substantially free of nuclease activity. By "substantially free of nuclease
15 activity" is meant that on admixture and incubation of an SSB preparation with target nucleic acid molecules under hybridization reaction conditions, more than 70 percent of the target nucleic acid molecules, and preferably more than 95 percent of the
20 target molecules, retain the capacity to hybridize with complementary polynucleotide molecules when measured by a hybridization procedure such as is described in Example 2. SSB substantially free of nuclease activity (isolated SSB) can be prepared by
25 the methods described in Example 1, or can be obtained from the commercial vendors described above.

Where SSB is to be utilized in a hybridization method in which a hybridization reaction admixture containing SSB is subjected to a temperature in the
30 range of about 30 degrees centigrade (30C) to about 100C, it is preferred that the SSB is a heat-stable SSB. A "heat-stable SSB" as defined herein is an SSB that retains more than 90% of its pre-treatment capacity to bind ssDNA preferentially over dsDNA
35 irrespective of the nucleotide sequence of the ssDNA

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after exposure in a hybridization reaction admixture to 90 degrees C (90C) for 10 min, preferably after exposure to 95C for 10 min, and more preferably after exposure to 100C for 8 min. A preferred heat-stable SSB is Eco SSB, that retains about 90% of its capacity to bind ssDNA after exposure to 100 C for 8 min. Weiner et al, J. Biol. Chem., 250:1972-80 (1975).

10 2. Preparation of Polynucleotides

The term "polynucleotide" as used herein refers to a nucleic acid molecule comprised of a linear strand of two or more deoxyribonucleotides and/or ribonucleotides, preferable more than 3. The exact size will depend on many factors, which in turn depends on the ultimate hybridization conditions of use, as is well known in the art. Polynucleotides used in the present invention include primers, probes, and the like. The term "primer" as used herein refers to a polynucleotide, whether purified from a nucleic acid restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a template nucleic acid strand is induced, i.e., in the presence of nucleotides and an agent for polymerization such as DNA polymerase, reverse transcriptase and the like, and at a suitable temperature and pH.

30 The term "probe" as used herein refers to a polynucleotide, whether purified from a nucleic acid restriction digest or produced synthetically, which is about 8 to 200 nucleotides in length, having a nucleotide base sequence that is substantially complementary to a predetermined specific nucleic

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acid-sequence present in a gene of interest, i.e. a target nucleic acid.

The polynucleotide must be sufficiently long to be capable of hybridizing under hybridizing conditions to a specific, preselected nucleic acid sequence. Where the polynucleotide is to function as a primer, it must also be sufficiently long to prime synthesis of extension products in the presence of agents for polymerization. The exact length of the polynucleotide therefore will depend on many factors, including hybridization temperature and the nucleotide sequence of the probe. For example, depending on the complexity of the target sequence, a polynucleotide typically contains 15 to 25 or more nucleotides, although it can contain fewer nucleotides. As few as 8 nucleotides in a polynucleotide have been reported as effective for use. Studier et al, Proc. Natl. Acad. Sci. USA, 86:6917-21 (1989). Short polynucleotides generally require lower temperatures to form sufficiently stable hybrid complexes with target.

The primers used herein are selected to be "substantially" complementary to the different strands of each specific sequence to be synthesized or amplified. This means that the primer must contain at its 3' terminus a nucleotide sequence sufficiently complementary to nonrandomly hybridize with its respective template strand. Therefore, the primer sequence may not reflect the exact sequence of the template. For example, a non-complementary polynucleotide can be attached to the 5' end of the primer, with the remainder of the primer sequence being substantially complementary to the strand. Such noncomplementary polynucleotides might code for an endonuclease restriction site or a site for

protein binding. Alternatively, noncomplementarity bases or longer sequences can be interspersed into the primer, provided the primer sequence has sufficient complementarity with the sequence of the strand to be synthesized or amplified to non-randomly hybridize therewith and thereby form an extension product under polynucleotide synthesizing conditions.

Sommer et al, Nuc. Acid Res., 17:6749 (1989), reports that primers having as little as a 3 nucleotide exact match at the 3' end of the primer were capable of specifically initiating primer extension products, although less nonspecific hybridization occurs when the primer contains more nucleotides at the 3' end having exact complementarity with the template sequence. Therefore, a substantially complementary primer as used herein must contain at its 3' end at least 3 nucleotides having exact complementarity to the template sequence. A substantially complementary primer preferably contains at least 10 nucleotides, more preferably at least 18 nucleotides, and still more preferably at least 24 nucleotides, at its 3' end having the aforementioned complementarity. Still more preferred are primers whose entire nucleotide sequence have exact complementarity with the template sequence.

The choice of a primer's nucleotide sequence depends on factors such as the distance on the nucleic acid from the region coding for the desired specific nucleic acid sequence present in a nucleic acid of interest and its hybridization site on the nucleic acid relative to any second primer to be used.

Polynucleotides can be prepared by a variety of methods including de novo chemical synthesis of

polynucleotides and derivation of nucleic acid fragments from native nucleic acid sequences existing as genes, or parts of genes, in a genome, plasmid, or other vector, such as by restriction endonuclease digest of larger double-stranded nucleic acids and strand separation or by enzymatic synthesis using a nucleic acid template.

De novo chemical synthesis of a polynucleotide can be conducted using any suitable method, such as, for example, the phosphotriester or phosphodiester methods. See Narang et al, Meth. Enzymol., 68:90, (1979); U.S. Patent No. 4,356,270; Itakura et al, Ann. Rev. Biochem., 53:323-56 (1989); and Brown et al, Meth. Enzymol., 68:109, (1979).

Derivation of a polynucleotide from nucleic acids involves the cloning of a nucleic acid into an appropriate host by means of a cloning vector, replication of the vector and therefore multiplication of the amount of the cloned nucleic acid, and then the isolation of subfragments of the cloned nucleic acids. For a description of subcloning nucleic acid fragments, see Maniatis et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, pp 390-401 (1982); and see U.S. Patents No. 4,416,988 and No. 4,403,036.

3. Hybridization Methods and Preparation of Primed Templates

In one embodiment the present invention contemplates an improved method for hybridizing a polynucleotide to a target sequence present on a nucleic acid to form a hybridization product. A hybridization reaction mixture is prepared by admixing effective amounts of a polynucleotide probe, a target nucleic acid and other components compatible

with a hybridization reaction including an effective amount of a single-stranded nucleic acid binding protein (SSB) but not including E. coli RecA protein, i.e., the hybridization reaction mixture is

5 substantially free of E. coli RecA protein. By substantially free in this context is meant that there is less than 10 ug of RecA, more preferably 1 ng of RecA protein per ml and still more preferably means that there is no detectable RecA protein by

10 standard immunological detection means.

In another improved method for hybridizing a primer to a template sequence present on a nucleic acid to form a primer-template hybridization product (primed template).

15 The primary purpose in including SSB in the hybridization is to reduce nonspecific hybridization reactions. Because nonspecific hybridization occurs more frequently as the complexity of the target nucleic acid increases the benefits of including SSB

20 in probe-target hybridization reaction admixtures increase as the complexity of the target increases. Thus, the method of the present invention is particularly useful where the target nucleic acid has a complexity comparable to that of lambda

25 bacteriophage DNA. The benefits are even more pronounced where the complexity of target is comparable to a procaryotic or mammalian genome.

Target nucleic acid sequences to be hybridized in the present methods can be present in any nucleic acid-containing sample so long as the sample is in a

30 form, with respect to purity and concentration, compatible with nucleic acid hybridization reaction. Isolation of nucleic acids to a degree suitable for hybridization is generally known and can be

35 accomplished by a variety of means. For instance,

nucleic acids can be isolated from a variety of nucleic acid-containing samples including body tissue, such as skin, muscle, hair, and the like, and body fluids such as blood, plasma, urine, amniotic fluids, cerebral spinal fluids, and the like. See, 5 for example, Maniatis et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (1982); and Ausubel et al, Current Protocols in Molecules Biology, John Wiley and Sons (1987).

10 The hybridization reaction mixture is maintained in the contemplated method under hybridizing conditions for a time period sufficient for the polynucleotide probe to hybridize to complementary nucleic acid sequences present in the 15 sample to form a hybridization product, i.e., a complex containing probe and target nucleic acid.

The phrase "hybridizing conditions" and its grammatical equivalents, when used with a maintenance time period, indicates subjecting the hybridization 20 reaction admixture, in the context of the concentrations of reactants and accompanying reagents in the admixture, to time, temperature and pH conditions sufficient to allow the polynucleotide probe to anneal with the target sequence, typically 25 to form a nucleic acid duplex. Such time, temperature and pH conditions required to accomplish hybridization depend, as is well known in the art, on the length of the polynucleotide probe to be hybridized, the degree of complementarity between the 30 polynucleotide probe and the target, the guanidine and cytosine content of the polynucleotide, the stringency of hybridization desired, and the presence of salts or additional reagents in the hybridization reaction admixture as may affect the kinetics of 35 hybridization. Methods for optimizing hybridization

conditions for a given hybridization reaction admixture are well known in the art.

Typical hybridizing conditions include the use of solutions buffered to pH values between 4 and 9, and are carried out at temperatures from 18 degrees C (18C) to 75C, preferably about 37C to about 65C, more preferably about 54C, and for time periods from 0.5 seconds to 24 hours, preferably 2 min.

Hybridization can be carried out in a homogeneous or heterogeneous format as is well known. The homogeneous hybridization reaction occurs entirely in solution, in which both the polynucleotide and the nucleic acid sequences to be hybridized (target) are present in soluble forms in solution. A heterogeneous reaction involves the use of a matrix that is insoluble in the reaction medium to which either the polynucleotide or target nucleic acid is bound. For instance, the body sample to be assayed can be affixed to a solid matrix and subjected to in situ hybridization.

In situ hybridization is typically performed on a body sample in the form of a slice or section of tissue usually having a thickness in the range of about 1 micron to about 100 microns, preferably about 1 micron to about 25 microns and more preferably about 1 micron to about 10 microns. Such sample can be prepared using a commercially available cryostat.

Alternatively, a heterogeneous format widely used is the Southern blot procedure in which genomic DNA is electrophoresed after restriction enzyme digestion, and the electrophoresed DNA fragments are first denatured and then transferred to an insoluble matrix. In the blot procedure, a polynucleotide probe is then hybridized to the immobilized genomic nucleic acids containing complementary nucleic acid

(target) sequences.

Still further, a heterogeneous format widely used is a library screening procedure in which a multitude of colonies, typically plasmid-containing bacteria or lambda bacteriophage-containing bacteria, is plated, cultured and blotted to form a library of cloned nucleic acids on an insoluble matrix. The blotted library is then hybridized with a polynucleotide probe to identify the bacterial colony containing the nucleic acid fragments of interest.

Typical heterogeneous hybridization reactions include the use of glass slides, nitro-cellulose sheets, and the like as the solid matrix to which target-containing nucleic acid fragments are affixed.

Also preferred are the homogeneous hybridization reactions such as are conducted for a reverse transcription of isolated mRNA to form cDNA, dideoxy sequencing and other procedures using primer extension reactions in which polynucleotide hybridization is a first step. Particularly preferred is the homogeneous hybridization reaction in which a specific nucleic acid sequence is amplified via a polymerase chain reaction (PCR).

The use of a SSB in a hybridization reaction mixture reduces the degree of nonspecific hybridization of a polynucleotide to a target nucleic acid. Therefore hybridization conditions can be carried out at temperatures lower than normally required to maintain high stringency, i.e., high specificity, in the hybridization reaction.

Where the nucleic acid containing a target sequence is in a double-stranded (ds) form, it is preferred to first denature the dsDNA, as by heating or alkali treatment, prior to conducting the hybridization reaction. The denaturation of the

dsDNA can be carried out prior to admixture with a polynucleotide to be hybridized, or can be carried out after the admixture of the dsDNA with the polynucleotide, the SSB or both. Where the
5 polynucleotide itself is provided as a double-stranded molecule, it too can be denatured prior to admixture in a hybridization reaction mixture, or can be denatured concurrently therewith the target-containing dsDNA.

10 Although the preceding has described hybridization reactions useful to produce a single hybridization product containing a specific nucleic acid sequence, it is often useful to utilize a hybridization reaction in a method for detecting a
15 specific nucleic acid sequence.

4. Detection of a Specific Nucleic Acid Sequence

The present invention therefore also
20 contemplates a method for detecting a specific (preselected) nucleic acid sequence by first conducting the before-described hybridization reaction to form a hybridization product, and then detecting the presence of the formed hybridization
25 product, thereby detecting the presence of the specific nucleic acid sequence in a nucleic acid-containing sample.

A nucleic acid-containing sample can be a body tissue or body fluid, and can be prepared as
30 described before for hybridization reaction admixtures.

The detection of a hybridization product formed in the hybridization reaction can be accomplished by a variety of means. Although there
35 are preferred embodiments disclosed herein for

hybridization product detection, it is to be understood that other well known detection means readily apparent to one skilled in the art are suitable for use in the presently contemplated process and associated diagnostic system.

5 In one approach for detecting the presence of a specific nucleic acid sequence, the polynucleotide probe includes a label or indicating group that will render a hybridization product in which the probe is present detectable. Typically such labels include
10 radioactive atoms, chemically modified nucleotide bases, and the like.

Radioactive elements operatively linked to or present as part of a polynucleotide probe provide a
15 useful means to facilitate the detection of a hybridization product. A typical radioactive element is one that produces beta ray emissions. Elements that emit beta rays, such as ^3H , ^{14}C , ^{32}P , and ^{35}S represent a class of beta ray emission-producing
20 radioactive element labels. A radioactive polynucleotide probe is typically prepared by enzymatic incorporation of radioactively labeled nucleotides into a nucleic acid using DNA polymerase, and then the labeled nucleic acid is denatured to
25 form a radiolabeled polynucleotide probe.

Alternatives to radioactively labeled polynucleotide probes are polynucleotides that are chemically modified to contain metal complexing agents, biotin-containing groups, fluorescent
30 compounds, and the like.

One useful metal complexing agent is a lanthanide chelate formed by a lanthanide and an aromatic beta-diketone, the lanthanide being bound to the nucleic acid or polynucleotide via a chelate
35 forming compound such as an EDTA-analogue so that a

fluorescent lanthanide complex is formed. See U.S. Patents No. 4,374,120, and No. 4,569,790 and published Patent Applications No. EP0139675 and No. WO87/02708.

5 Biotin or acridine ester-labeled oligonucleotides and their use in polynucleotides have been described. See U.S. Patent No. 4,707,404, published Patent Application EP0212951 and European Patent No. 0087636. Useful fluorescent marker
10 compounds include fluorescein, rhodamine, Texas Red, NBD and the like.

 A labeled nucleotide present in a hybridization product renders the hybridization product itself labeled and therefore distinguishable
15 over other nucleic acids present in a sample to be assayed. Detecting the presence of the label in the hybridization product and thereby the presence of the hybridization product, typically involves separating the hybridization product from any labeled
20 polynucleotide probe that is not hybridized to a hybridization product.

 Techniques for the separation of single-stranded polynucleotide, such as non-hybridization labeled polynucleotide probe, from a hybridized
25 product are well known, and typically involve the separation of single-stranded from non-single-stranded nucleic acids on the basis of their chemical properties. More often separation techniques involve the use of a heterogeneous hybridization format in
30 which the non-hybridized probe is separated, typically by washing, from the hybridization product that is bound to a solid matrix. Exemplary is the Southern blot technique, in which the matrix is a nitrocellulose sheet and the label is ³²P. Southern,
35 J. Mol. Biol., 98:503 (1975).

- In another embodiment, the hybridization product detection step comprises detecting an amplified nucleic acid product. An amplified nucleic acid product is the product of an amplification process well known in the art that is referred to as the polymerase chain reaction (PCR).

5. Primer Extension Reactions

The primed template can be used to produce a strand of nucleic acid having a nucleotide sequence complementary to the template, i.e., a template-complement.

If the template whose complement is to be produced is in the form of double stranded nucleic acid, it is typically first denatured, usually by melting, into single strands, such as ssDNA. The nucleic acid is then subjected to a (first) primer extension reaction by treating (contacting) the nucleic acid with a (first) polynucleotide synthesis primer having as a portion of its nucleotide sequence a sequence selected to be substantially complementary to a portion of the sequence of the template. The primer is capable of initiating a primer extension reaction by hybridizing to a specific nucleotide sequence, preferably at least about 8 nucleotides in length and more preferably at least about 20 nucleotides in length. This is accomplished by mixing an effective amount of the primer with the template nucleic acid, an effective amount of SSB, and an effective amount of nucleic acid synthesis inducing agent to form a primer extension reaction admixture. The admixture is maintained under polynucleotide synthesizing conditions for a time period, which is typically predetermined, sufficient for the formation of a primer extension reaction

product.

The primer extension reaction is performed using any suitable method. Generally polynucleotide synthesizing conditions are those wherein the reaction occurs in a buffered aqueous solution, preferably at a pH of 7-9, most preferably about 8. Preferably, a molar excess (for genomic nucleic acid, usually about 10^6 :1 primer:template) of the primer is admixed to the buffer containing the template strand. A large molar excess is preferred to improve the efficiency of the process. For polynucleotide primers of about 20 to 25 nucleotides in length, a typical ratio is in the range of 50 ng to 1 ug, preferably 250 ng, of primer per 100 ng to 500 ng of mammalian genomic DNA or per 10 to 50 ng of plasmid DNA.

The deoxyribonucleotide triphosphates (dNTPs) dATP, dCTP, dGTP, and dTTP are also admixed to the primer extension reaction admixture in amounts adequate to support the synthesis of primer extension products, and depends on the size and number of products to be synthesized. The resulting solution is heated to about 90C - 100C for about 1 to 10 minutes, preferably from 1 to 4 minutes. After this heating period the solution is allowed to cool to room temperature, which is preferable for primer hybridization. To the cooled mixture is added an appropriate agent for inducing or catalyzing the primer extension reaction, and the reaction is allowed to occur under conditions known in the art. The synthesis reaction may occur at from room temperature up to a temperature above which the inducing agent no longer functions efficiently. Thus, for example, if DNA polymerase is used as inducing agent, the temperature is generally no

greater than about 40C unless the polymerase is heat-stable.

The inducing agent may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, E. coli, DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, T4 DNA polymerase, T7 DNA polymerase, recombinant modified T7 DNA polymerase, other available DNA polymerases, reverse transcriptase, and other enzymes, including heat-stable enzymes, which will facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to each nucleic acid strand.

Heat-stable DNA polymerases are particularly preferred as they are stable in a most preferred embodiment in which PCR is conducted in a single solution in which the temperature is cycled. Representative heat-stable polymerases are the DNA polymerases isolated from Bacillus stearothermophilus (Bio-Rad), Thermus thermophilus (FINZYME, ATCC #27634), Thermus species (ATCC #31674), Thermus aquaticus strain TV 1151B (ATCC #25105), Sulfolobus acidocaldarius, described by Bukhrashuili et al, Biochem. Biophys. Acta, 1008: 102-7 (1989) and by Elie et al, Biochem. Biophys. Acta, 951:261-7 (1988), and Thermus filiformis (ATCC #43280). Particularly preferred is Taq DNA polymerase available from a variety of sources including Perkin Elmer Cetus, (Norwalk, CT), Promega (Madison, WI) and Stratagene (La Jolla, CA), and AmpliTaq™ DNA polymerase, a recombinant Taq DNA polymerase available from Perkin-Elmer Cetus.

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- Generally, the synthesis will be initiated at the 3' end of each primer and proceed in the 5' direction along the template strand, until synthesis terminates, producing molecules of different lengths.

5 There may be inducing agents, however, which initiate synthesis at the 5' end and proceed in the above direction, using the same process as described above.

The primer extension reaction product can then be subjected to a second primer extension reaction by
10 treating it with a second polynucleotide synthesis primer having a preselected nucleotide sequence. The second primer is capable of initiating the second reaction by hybridizing to a nucleotide sequence, preferably at least about 8 nucleotides in length and
15 more preferably at least about 20 nucleotides in length, found in the first product. This is accomplished by mixing the second primer, preferably a predetermined amount thereof, with the first product, preferably a predetermined amount thereof,
20 to form a second primer extension reaction admixture. The admixture is maintained under polynucleotide synthesizing conditions for a time period, which is typically predetermined, sufficient for the formation of a second primer extension reaction product.

25 In preferred strategies, the first and second primer extension reactions are the first and second primer extension reactions in a polymerase chain reaction (PCR).

PCR is carried out by simultaneously cycling,
30 i.e., performing in one admixture, the above described first and second primer extension reactions, each cycle comprising polynucleotide synthesis followed by denaturation of the double stranded polynucleotides formed. Methods and systems
35 for amplifying a specific nucleic acid sequence are

described in U.S. Patents No. 4,683,195 and No. 4,683,202, both to Mullis et al; U.S. Patent No. 4,889,818 to Gelfand et al.; and the teachings in PCR Technology, Erlich, ed., Stockton Press (1989);

- 5 Faloona et al, Methods in Enzymol., 155:335-50 (1987); and Polymerase Chain Reaction, Erlich et al, eds., Cold Spring Harbor Laboratories Press (1989).

10 In one embodiment, a method of amplifying a specific double-stranded nucleic acid sequence in a nucleic acid sample is contemplated. The method comprises the steps of:

(a) Separating the strands of the double-stranded nucleic acid molecule to form single-stranded templates.

- 15 (b) Treating the single-stranded templates with the following in amounts effective to induce polynucleotide synthesis under polynucleotide synthesizing conditions: (i) primers that are selected so as to be substantially complementary to portions of the different strands of the specific
20 double-stranded sequence to hybridize therewith such that an extension product synthesized from one primer, when it is separated from its template (complementary) nucleic acid strand, can serve as a
25 template for synthesis of the extension product of the other primer, (ii) an SSB, preferably Eco SSB, and (iii) an enzyme capable of producing primer extension products from said single-stranded templates, wherein said treatment is conducted under
30 conditions such that the primers hybridize to the specific sequences and an extension product of each primer is synthesized by the action of the enzyme, said product being complementary to each single-stranded template.

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- (c) Separating the primer extension products formed in step (b) from the templates on which they are synthesized to produce single-stranded molecules.

(d) Treating the single-stranded molecules produced in step (c) with effective amounts of the primers, SSB and enzyme of step (b) under conditions such that a primer extension product is synthesized using the single-stranded molecules formed in step (c) as a template.

10 PCR is carried out by cycling i.e., simultaneously performing in one admixture, the above described: 1) denaturing step to form single-stranded templates, 2) hybridization step to hybridize primer to ss template, and 3) primer extension steps to form
15 the extended product. PCR is performed in the above sequence (cycle) by changing the temperature of the PCR mixture to a temperature compatible with each step, in series.

The primer extension reaction conditions
20 involve maintaining the reaction mixture for a time period and at a temperature sufficient for a DNA polymerase primer extension reaction to occur to produce primer extension products as is well known. Conditions for conducting a primer extension reaction
25 are well known. In a PCR format, the maintenance is carried out quickly to conveniently facilitate numerous cycles, in about 1 second to 5 minutes, preferably about 1.5 minutes, and at about 40C to 75C, preferably about 72C. Conducting at least one
30 cycle of PCR results in the formation of amplified nucleic acid products. The PCR is typically conducted with at least 15 cycles, and preferably with about 20 to 40 cycles.

Hybridizing conditions were described earlier
35 and are suitable for use in the PCR format. However,

it is preferred and convenient to conduct hybridization in short periods of time, in 5 seconds to 12 minutes, preferably in 2 minutes, and in the temperature range of 30C to 75C, preferably about 40C to 65C, and more preferably about 54C.

A suitable SSB for use in a hybridization reaction mixture is one of the class of SSBs described previously, preferably a nuclease free, heat-stable SSB, and more preferably is Eco SSB. Exemplary hybridization reaction mixtures and reaction conditions are described further in Example 2.

In preferred embodiments, detecting the presence of any primer extension product formed in step (d) thereby provides a method for detecting the presence of a specific double-stranded nucleic acid sequence in the sample.

Detection of amplified nucleic acid product can be accomplished by any of a variety of well known techniques. In a preferred embodiment, the amplified product is separated on the basis of molecular weight by gel electrophoresis, and the separated products are then visualized by the use of nucleic acid specific stains which allow one to observe the discreet species of resolved amplified product present in the gel. Although numerous nucleic acid specific stains exist and would be suitable to visualize the electrophoretically separated nucleic acids, ethidium bromide is preferred.

Alternative methods suitable to detect the amplified nucleic acid product include hybridization-based detection means that use a labeled polynucleotide probe capable of hybridizing to the amplified product. Exemplary of such detection means include the Southern blot analysis, ribonuclease

protection analysis using in vitro labeled polyribonucleotide probes, and the like methods for detecting nucleic acids having specific nucleotide sequences. See, for example, Ausubel et al., Current
5 Protocols in Molecular Biology, John Wiley & Sons, 1987.

In one approach for detecting the presence of a specific nucleic acid sequence, the deoxyribonucleotide triphosphates (dNTPs) used in the
10 primer extension reaction include a label or indicating group that will render a primer extension product detectable. Typically such labels include radioactive atoms, chemically modified nucleotide bases, and the like.

15 A labeled nucleotide residue present in a nucleic acid renders the nucleic acid itself labeled and therefore distinguishable over other nucleic acids present in a sample to be assayed. Detecting the presence of the label in the nucleic acid and
20 thereby the presence of the specific nucleic sequence, typically involves separating the nucleic acid from any labeled dNTP that is not present as part of a primer extension reaction product.

Numerous applications of the PCR-based
25 amplification method are contemplated that will be readily apparent to one skilled in the art. For example, cloning mRNA through reverse transcription to produce cDNA can be made more sensitive by the use of PCR-based amplification of the produced cDNA.
30 Insofar as nucleic acid sequencing can be conducted on PCR-amplified nucleic acid, the present invention can be used to improve the amplified nucleic acids production step in a sequencing procedure. A variety of the other recombinant DNA cloning and nucleic acid
35 manipulative steps have been described that involved

PCR.- See, for example, PCR Technology, Erlich, ed., Stockton Press (1989), and Polymerase Chain Technology, Erlich et al, eds., Cold Spring Harbor Laboratory Press (1989).

5 In a related embodiment, the present invention contemplates the application of the improved PCR amplification method to samples of nucleic acid in which the nucleic acid is hybridized and primer extension products are formed from a hybridization
10 product comprised of triple-stranded nucleic acid molecules. Triple-stranded nucleic acid molecules, or triple helixes, have been produced in vitro by hybridization of polynucleotides and duplex DNA. Moser et al, Science, 238:645-50 (1987); Kohwi et al,
15 Proc. Natl. Acad. Sci. USA, 85:3781-85 (1988); Wells et al, FASEB J., 2:2939-49 (1988); Htun et al, Science, 243:1571-76 (1989); and Rajagopal et al, Nature, 339:637-40 (1989). Therefore although a preferred template for hybridization and primer
20 extension in a PCR method is a single-stranded nucleic acid, the invention contemplates the use of a double-stranded template to form a triple-stranded hybridization product.

 In practicing the methods of the present
25 invention to form a triple-stranded hybridization product, it is to be understood that the initial denaturation of double-stranded nucleic acids to form single-stranded template is not required. Instead, the hybridization reaction mixture can be used to
30 treat double-stranded template rather than single-stranded template forming a triple helix hybridization product.

- C. Compositions

Also contemplated by the present invention is a composition for producing a template-complement from a nucleic acid template that contains a specific nucleic acid sequence according to the methods of the present invention. The composition comprises, in amounts sufficient to produce at least one template-complement, (a) an isolated single-stranded nucleic acid binding protein, and (b) a polynucleotide primer selected so as to be sufficiently complementary to a portion of a nucleic acid template containing said specific nucleic acid sequence to hybridize therewith such that a primer extension product can be synthesized therefrom in a primer extension reaction.

In another embodiment, a composition for producing a template-complement comprises effective amounts of (a) an isolated single-stranded nucleic acid binding protein, and (b) an inducing agent for nucleic acid synthesis by primer extension. A preferred inducing agent is one of the previously described DNA polymerase enzymes suitable for the synthesis of primer extension products.

In one embodiment for amplifying a specific nucleic acid via the PCR, composition comprises, in effective amounts, (a) an isolated single-stranded nucleic acid binding protein, and (b) two polynucleotide primers that are selected so as to be substantially complementary to portions of different strands of a double-stranded nucleic acid containing said specific nucleic acid sequence to hybridize therewith such that an extension product synthesized from one primer, when it is separated from its complementary nucleic acid strand, can serve as a template for synthesis of the extension product of

the other primer.

In all embodiments of a contemplated composition additional reagents can also be included such as a divalent cation, preferably Mg^{++} , Mn^{++} , Ca^{++} , or Zn^{++} , and preferably an amount of divalent cation sufficient to produce a primer extension reaction mixture having an effective amount of divalent cation concentration in the range of 0.5 mM to 20 mM. Where the composition is formulated for use in PCR-based methods, it is preferred that the divalent cation is Mg^{++} and the effective amount is about 1.5 mM.

The single-stranded binding protein (SSB) for inclusion in a composition of this invention is one of the SSBs described herein for practicing a contemplated method. The amount of SSB to be included in a system is an amount sufficient to produce a reaction mixture having an effective amount of SSB in the range of 1 ng to 10 ng per 100 ng of nucleic acid, and more preferably is about 0.25 ug to 2 ug of SSB per 100 ng of nucleic acid.

A particularly preferred composition for use in PCR includes isolated Eco SSB, Mg^{++} in an amount sufficient to produce a reaction mixture concentration of about 1.5 mM, and a heat-stable DNA polymerase, preferably Taq polymerase. An additional reagent for this embodiment can be two polynucleotide primers selected having the capacity to function in PCR as described above.

In another embodiment the present invention contemplates an SSB/PCR composition for enhancing PCR amplification specificity, i.e., a composition that increases the ratio of primary to secondary (mismatch) PCR products. The composition comprises, in aqueous solution, SSB in an amount sufficient to enhance the production of primary polymerase chain

reaction products, and preferably reduce the formation of secondary PCR products. The SSB/PCR solution also contains a non-ionic detergent in an amount sufficient to stabilize Taq polymerase (U.S. Patent No. 4,889,818) against degradation during polymerase chain reaction amplification, i.e., it degrades less than 1 weight percent, preferably less than 0.1 weight percent and more preferably less than 0.01 weight percent DNA at 37°C over 16 hours. The SSB/PCR solution is also characterized as being free of nuclease activity.

Typically, the SSB/PCR solution contains biologically active SSB at a concentration of at least 0.01 milligrams per milliliter (mg/ml), preferably about 0.1 mg/ml, and more preferably about 0.5 mg/ml. Usually the concentration of SSB is no more than about 20 milligrams per milliliter. Preferred SSBs are Eco SSB, T4 gene 32 protein, T7 SSB, and the like.

The SSB/PCR composition preferably also includes a carrier protein in an amount sufficient to stabilize the SSB and/or inhibit PCR inhibitors. Usually the carrier protein is present at a concentration of at least 0.05 milligrams per milliliter and typically is in the range of 0.05 to 0.15 milligrams per milliliter, and preferably is about 0.1 milligrams per milliliter. Proteins useful for stabilizing biologically active proteins in solution are well known in the art. A preferred carrier protein is serum albumin such as bovine serum albumin (BSA), and the like. Acetylated BSA, prepared according to the method of Gonzalez et al., (1972) Arch. Biochem. Biophys., 182:404-408, is preferred.

- Preferably, the SSB/PCR composition further includes a chelating agent at a concentration sufficient to provide a cation-chelating capacity corresponding to that which 0.5 to 1.5 ethylenediaminetetraacetic acid (EDTA) would provide in the composition.

In another preferred embodiment, the SSB/PCR composition includes a buffering agent at a concentration sufficient to provide a buffering capacity corresponding to that which 0.05 to 500 mM tris(hydroxymethyl)-aminomethane would provide in the composition.

Buffering agents other than tris(hydroxymethyl)aminomethane can be used, but they must be present in the solution at a concentration that has the capacity to resist a change in pH value upon the addition of either H^+ or OH^- in a manner that is similar to that of 0.05 to 500 mM tris(hydroxymethyl)aminomethane. A combination of buffering agents can be used, so long as the solution has the required buffering capacity. Methods for determining the buffering capacity of a solution are well known in the art.

Of course, the comparison of buffering capacity is carried out in the presence of the salt to be used, at the salt concentration to be used, and with the solutions being compared at about the same temperature, preferably at a temperature within the range of about 15°C to about 25°C. Other exemplary buffering agents include sodium acetate, and the like.

In preferred embodiments, the composition further includes a salt at a concentration sufficient to maintain the solubility of the SSB in the composition. Typical salts include NaCl, KCl, and

the like. Usually the salt is present at a concentration of at least 300 mM and more preferably at a concentration of at least 500 mM, the salt concentration being determined using the entire salt formula as opposed to a formula representing an ionized form of the salt.

The SSB/PCR composition of the present invention also preferably includes an anti-freezing agent at a concentration sufficient to prevent freezing of the composition at at least -30 degrees centigrade. Typical anti-freezing agents include glycerol, polyethylene glycol and the like. Usually the anti-freezing agent is present in the composition at at least 30 volume percent and more preferably is present at a concentration of at least 50 volume percent.

The SSB/PCR composition of the present invention also preferably includes a reducing agent present at a concentration sufficient to provide a reducing capacity corresponding to that which 0.5 to 2 mM 2-mercaptoethanol would provide in the composition.

Typically the composition will have a pH value sufficient to maintain the biological activity of the SSB. Usually that pH value will be in the range of 7 to 8 and is preferably about 7.5. The composition is preferably free of nucleic acid, i.e., contains less than 0.01 weight percent nucleic acid.

In preferred embodiments the SSB/PCR composition contains at least 0.1 volume percent non-ionic detergent, preferably at least 0.2 volume percent, and more preferably at least 1.0 volume percent non-ionic detergent. Preferred non-ionic detergents include octylphenoxy polyethoxy ethanol, polyoxyethylene sorbitan monolaurate, polyoxyethylene

lauryl ether, and the like.

The compositions can be packaged in kit form. As used herein, the term "package" refers to a solid matrix or material customarily utilized in a system and capable of holding within fixed limits one or more of the reagent components for use in a method of the present invention. Such materials include glass and plastic (e.g., polyethylene, polypropylene and polycarbonate) bottles, vials, paper, plastic and plastic-foil laminated envelopes and the like. Thus, for example, a package can be a glass vial used to contain the appropriate quantities of polynucleotide primer(s), isolated SSB, a divalent cation and a DNA polymerase, or a combination thereof.

In embodiments where the system is utilized diagnostically to detect the presence of a specific nucleic acid, it is preferred that the diagnostic system further includes a label or indicating means capable of signaling the formation of a complex containing a polynucleotide by the methods of the present invention.

In preferred embodiments, the indicating means comprises a probe, the probe being operatively linked to a label, thereby providing a means to detect an amplified nucleic acid product formed. Preferred labels are those discussed hereinbefore, especially ^{32}P and ^{35}S for polynucleotide probes.

Kits useful for producing a template-complement or for amplification or detection of a specific nucleic acid sequence using a primer extension reaction methodology also typically include, in separate containers within the kit, dNTPs where N is adenine, thymine, guanine and cytosine, and other like agents for performing primer extension reactions.

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- The reagent species, indicating means or primer extension reaction reagents of any system described herein can be provided in solution, as a liquid dispersion or as a substantially dry power, e.g., in lyophilized form. Where the reagent species or indicating means is an enzyme, the enzyme's substrate can also be provided in a separate package of a system. A solid support and one or more buffers can also be included as separately packaged elements in this system.

Examples:

The following examples are given for illustrative purposes only and do not in any way limit the scope of the invention.

1. Purification of Single-Stranded Nucleic Acid Binding Protein

Single-stranded nucleic acid binding protein (Eco SSB) was purified from E. coli strain K12-H1-TRP/pTL119A-5 essentially as previously described by Lohman et al., Biochem., 25: 21-25 (1986), according to the following procedure.

A. Cell Growth and Induction

E. coli strain K12-H1-TRP/pTL119A-5 was grown at 30 degrees centigrade (30C) in media containing 10 grams per liter (g/l) bactotryptone, 5 g/l yeast extract, 5 g/l NaCl and 2 ml/l of a solution containing 50 mg/ml thiamine. Single colony isolates of SSB-coli were admixed with 100 mls of media and grown overnight at 30C to produce an overnight culture. 5 ml of the overnight culture was admixed per 1 liter of media in a 2 liter flask and the admixture maintained at 30C until the bacterial

cells had grown to an optical density at 600 nanometers (O.D. 600) of 0.4 to 0.6 to produce a log-phase culture. 18 liters of log-phase culture were produced by this procedure.

5 The log-phase culture was then heat shocked by maintaining the culture at 42C for thirty minutes. The heat shocked culture was maintained for four to five hours at 37C. Thereafter culture was placed on ice and the bacterial cells collected from the
10 culture by centrifugation at 5,000 rpm in a J-6B rotor (Beckman Instruments, Palo Alto, CA) for ten minutes at 4C. All buffers and procedures utilized hereafter were at 4C unless otherwise noted. The resulting bacterial cell pellet was resuspended in a
15 0.5 liter solution containing 10 mM Tris-HCl at pH 8.0, 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 M NaCl. The resuspended pellet was centrifuged for ten minutes at 5,000 rpm in the same rotor at 4C, and the resulting cell pellet was resuspended in 500
20 ml of a lysis solution containing 50 mM Tris-HCl at pH 8.3, 0.2 M NaCl, 15 mM spermidine trichloride, 1 mM EDTA and 10% sucrose. The resulting cell pellet suspension was frozen by addition drop by drop to liquid nitrogen and stored at -70C until used.

25

B. Cell Lysis

 The frozen bacterial cells were thawed overnight to 0C in a freezer. The cells were then thawed in a warm water bath with gentle mixing at
30 slightly below 10C. Freshly prepared 0.1 M phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 0.1 mM. A solution containing 10 mg/ml of lysozyme was added to the solution to a final concentration of 200 ug/ml. The resulting
35 solution was maintained at 4C for thirty minutes. A

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freshly prepared solution of 4% sodium deoxycholate in H₂O was admixed to the solution to a final concentration of 0.05%. The resulting solution was maintained at 15C for thirty minutes to lyse the bacterial cells and produce a lysate. This lysate was sonicated for three minutes at 50% duty cycle on a power setting of 10 using a model 250 sonicator manufactured by Branson (Danbury, CT). The lysate was then centrifuged in a SA 600 rotor (Sorval, Dupont, Wilmington, DE) at 14,000 rpm for sixty minutes at 4C. The resulting supernatant was collected and termed fraction I.

C. Purification of Cell Lysate

A solution containing 10% Polymin P (PEI; polyethylenimine) at pH 6.9 (Sigma Chemical Corp., St. Louis, MO) was slowly admixed with 250 ml of fraction I to a final concentration of 0.4%. The resulting solution was stirred gently for fifteen minutes to precipitate protein. The precipitate was collected by centrifugation in 60 ml capped tubes in a SA 600 rotor at 14,000 rpm for twenty minutes at 4C. The resulting pellet was gently resuspended in 250 ml of a solution containing 50 mM Tris-HCl at pH 8.3, 1 mM EDTA, 0.4 M NaCl and 20% glycerol by first gently breaking the pellets with a tissue homogenizer and then slowly stirring the solution at 4C for thirty minutes. After the pellet appears to be completely resuspended, the solution is stirred for an additional fifteen minutes at 4C. The insoluble debris is then removed by centrifugation at 14,000 rpm in a SA 600 rotor for twenty minutes at 4C. The resulting supernatant is collected and termed fraction II.

- Solid ammonium sulfate was slowly added over a thirty minute period to fraction II with constant stirring at 4C until approximately 27% saturation with ammonium sulfate was reached. This solution was stirred for an additional thirty minutes at 4C and then centrifuged for thirty minutes in 60 ml capped tubes at 14,000 rpm in a SA 600 rotor at 4C. The resulting pellets were gently resuspended in 225 ml of a solution containing 50 mM Tris-HCl at pH 8.3, 1 mM EDTA, 0.3 M NaCl and 20% glycerol. After the pellet is completely resuspended the solution is centrifuged at 14,000 rpm for twenty minutes at 4C in a SA 600 rotor to remove any insoluble material. The resulting supernatant is termed fraction III.

Fraction III containing approximately 225 mg protein in 225 ml was dialyzed against 4 liters of dialysis solution containing 50 mM Tris HCl at pH 8.3, 1 mM EDTA, 150 mM NaCl and 20% glycerol for 12 to 18 hours. A portion of the dialyzed fraction III containing about 50 mg protein was diluted in the dialysis solution until its conductivity was brought to within 10% of the conductivity of dialysis solution, and was then applied to a 5.0 X 10.0 cm P-11 phosphocellulose column (Whatman Inc., Clifton, NJ) that had been previously activated according to the manufacturer's instructions and equilibrated with dialysis solution. The dialyzed and diluted fraction III was loaded onto the P-11 phosphocellulose column at a flow rate of 6 ml/minute. The phosphocellulose column was washed with 400 ml of column buffer containing 50 mM Tris-HCl at pH 8.3, 1 mM EDTA, 150 mM NaCl and 20% glycerol to remove any proteins that did not bind to the phosphocellulose column. Proteins that bound the P-11 phosphocellulose column were eluted with a linear gradient of KCl ranging

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from a concentration of 0 M to 2.0 M in column buffer. Fractions containing 10 ml of eluate were collected and the optical density at 280 nm measured. Peak fractions were pooled and dialyzed against a 4 liter solution containing 20 mM Tris-HCl, pH 8.3, 1 mM EDTA, 500 mM NaCl, 1 mM beta-mercaptoethanol and 50% glycerol and stored at -20C until used. The resulting solution contained Eco SSB protein at a concentration of 1 mg per ml and was free of nuclease contamination.

2. Detection of Specific Nucleic Acid Sequences in Genomic DNA using the Polymerase Chain Reaction /

Genomic DNA was purified by phenol extraction and alcohol precipitation according to the procedures of Ausubel et al, Current Protocols in Molecular Biology, John Wiley and Sons (1987), from blood obtained by tail bleed from a transgenic mouse having about 1 to 2 copies of a lambda transgene vector, and is referred to as lambda transgene mouse genomic DNA. Genomic DNA was also obtained from human white blood cells by the procedure of Miller et al, Nuc. Acid Res., 16:1215 (1988), and referred to as human genomic DNA.

Polynucleotide primers having the deoxyribonucleotide sequences shown in Table 1 below were prepared by chemical synthesis using a model 381A polynucleotide synthesizer (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's instructions.

Table 1

Polynucleotide		Nucleotide Base Sequence	
<u>Designation</u>			
5	pr 886	5'-TCTGGCTCCAGCCAAAGCCACCCTAG-3'	
	pr 887	5'-GGCTGAGCCCAGTGCCTCCTTGAGTA-3'	
	pr 4266	5'-GGTGGCGACGACTCCTGGAGCCC-3'	
	pr 19012	5'-GACAGTCACTCCGGCCCCGTGCGG-3'	
	pr 1A	5'-TGTAACACGACGGCCAGTGGGGCGGCC ACAATTTTCGCGCCAAACTTGACCG-3'	
10	pr 1B	5'-CAGGAAACAGCTATGACCGTGGGCAGC CTGCGCCCCGTTTGGGTCC-3'	
	pr 2A	5'-TGTAACACGACGGCCAGTCAAGGGATA ATGTTTCGAACGCTGTTT-3'	
	pr 2B	5'-CAGGAAACAGCTATGACCCCCAGATTA ACACGGAAACTTTCCATTTA-3'	
15	pr 737	5'-CGACGACTCGTGGAGCCC-3'	
	pr 738	5'-GACAGTCACTCCGGCCCCG-3'	

A hybridization reaction admixture was formed
 by combining the following reagents in a sterile 0.5
 milliliter (ml) microfuge tube: (a) 69 microliters
 (ul) of sterile, autoclaved H₂O; (b) 10 ul of 10x
 reaction buffer containing 500 mM KCl, 100 mM Tris-
 HCl (pH 8.3), 15 mM MgCl₂, and 0.1% sterile gelatin,
 (c) 8 ul of a solution containing 2.5 mM each of the
 deoxynucleotidetriphosphates (dNTP's) dGTP, dCTP,
 dTTP and dATP; (d) 2 ul of a solution containing 250
 ng each of two polynucleotide primers, selected from
 Table 1 and being indicated in the legends to Figures
 1-4, (e) 10 ul of DNA template dilution buffer (10 mM
 Tris-HCl, pH 8.0, 1 mM EDTA, 10 mM NaCl) containing
 genomic DNA as template, the particular DNA sample
 and amount being indicated in the legends to Figures
 1-4; and (f) zero to 1.0 ul (as indicated) of a
 solution containing 1 milligram (mg) per ml of Eco
 SSB protein prepared in Example 1 and free of

nuclease contamination.

The hybridization reaction admixture was heated to 94C and maintained at 94C for 3 min to denature the duplex DNA (genomic DNA) present and
5 form single-stranded templates. Then the admixture was cooled to 54C and maintained at 54C for 3 min to allow hybridization to occur and form duplex DNA. The hybridized admixture was then centrifuged for 10 seconds, in a microfuge at about 12,000 x g to
10 collect condensation off the microfuge tube walls, and 0.5 ul of a solution containing 2.5 units of either Taq DNA polymerase or AmpliTaq™ DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT) was admixed to form a primer extension reaction
15 admixture.

Each microfuge tube containing the above-prepared primer extension reaction admixture was overlaid with 50 ul of mineral oil and then placed into a DNA Thermal Cycler (Perkin-Elmer Cetus,
20 Norwalk, CT) and subjected to the following temperature and time conditions: (1) 72C for 1.5 min to allow for primer extension, (2) 91C for 1 min to heat denature the duplex DNA and (3) 54C for 2 min to allow single-stranded nucleic acids to hybridize.
25 Thereafter the same tubes were subjected to further cycles of conditions (1), (2) and (3) for a total of 30 cycles according to the manufacturer's instructions. The cycled tubes were then maintained at 72C for 10 min, and then maintained at 4C for 12
30 hours.

The contents of each primer extension reaction admixture was then analyzed on 6% polyacrylamide gels in 1x TBE by loading 35 ul of admixture sample and 5 ul of 10x sample buffer onto an 8 centimeter gel,
35 electrophoresing the gel at 100v for about 1 hr, and

staining the electrophoresed gel with ethidium bromide to visualize the electrophoresed nucleic acids.

The sensitivity of a PCR nucleic acid detection system using Eco SSB was determined using the above-described procedures and with the following additional details. The hybridization reaction admixture was prepared containing varying amounts of lambda transgene mouse genomic DNA, from 0.001 ng to 1000 ng. Carrier DNA comprising calf thymus DNA (100 ng) was also admixed to each tube containing less than 100 ng of genomic DNA. Duplicate sets of hybridization reaction admixtures were prepared with the varying amounts of genomic DNA; one set contained 250 ng (0.25 ul) of Eco SSB and the other set did not contain Eco SSB. The polynucleotide primers used were pr 4266 and pr 19012, whose sequences are shown in Table 1. Taq DNA polymerase was admixed in each tube and the PCR reaction mixtures were then subjected to PCR cycles as described above. The results of the sensitivity analysis are shown in Figures 1A and 1B.

By use of primers pr 4266 and pr 19012, an amplified nucleic acid fragment comprising a duplex DNA molecule that corresponds in sequence to the target specific nucleic acid is detected having a size of about 520 base pairs (bp), and is indicated in Figures 1A and 1B by the arrow. In the absence of Eco SSB (Figure 1A), the detected duplex DNA molecule was not a significant component above the level of detection of non-specific DNA molecules until at least 100 ng of template DNA were present in the hybridization and primer extension reaction admixtures. In the presence of 250 ng of Eco SSB, the detection of nonspecific DNA molecules was

substantially reduced and the limit of detection (sensitivity) for the target sequence was at least as low as 0.1 ng of template DNA (lane 3, Figure 1B).

The amount of Eco SSB to be added to a PCR nucleic acid detection system for effective improvement of detection was also determined by the above-described procedures. The hybridization reaction admixture was prepared using from 10 ng to 10,000 ng of Eco SSB in each tube, including AmpliTaq™ DNA polymerase, 100 ng of template lambda transgene mouse genomic DNA and the primers pr 4266 and pr 19012, and the PCR cycles were conducted as before. The results of varying the Eco SSB concentration are shown in Figure 2.

The results in Figure 2 show that Eco SSB is effective to improve PCR-based detection of specific nucleic acids over a wide range of Eco SSB protein concentrations, from below 10 ng to above 5 ug per 100 ul of hybridization reaction admixture. Particularly desirable results were obtained in which nonspecific bands were substantially reduced at concentrations of Eco SSB above 100 ng and below 2 ug per 100 ul of hybridization reaction admixture.

The requirements for stringency in the hybridization reaction were evaluated when Eco SSB is used in a PCR-based detection system by conducting the hybridization reaction at either 42C or 54C. The method was conducted as described above with the following changes. Hybridization reaction tubes were prepared containing (1) 100 ng of human genomic DNA and the primers pr 886 and pr 887, (2) 100 ng of lambda transgene mouse genomic DNA and the primers pr 4266 and pr 19012, or (3) 100 ng of the mouse DNA and the primers pr 737 and pr 738 (18-mers), and further containing either 250 ng of Eco SSB (+) or no Eco

SSB (-). Duplicate sets of the above tubes were prepared, each containing Taq DNA polymerase, and were subjected to the above PCR cycles in which the hybridization (annealing) temperature was either 42C or 54C. The results of the hybridization stringency determination are shown in Figure 3.

Using the mouse genomic DNA in combination with either the primer set pr 4266 and pr 19012 (each 23-mers) (lanes 5, 6, 11 and 12) or the primer set pr 737 and pr 738 (each 18-mers) (lanes 3, 4, 9 and 10) in the PCR system with Eco SSB, a specific duplex DNA of about 520 bp is detected (Figure 3, Lanes 4, 6, 10 and 12). Using the human genomic DNA in combination with the primer set pr 886 and pr 887 in the PCR system with Eco SSB, a specific duplex DNA of about 1800 bp is detected (lanes 7 and 8). However, in the absence of Eco SSB, the specific sequences are either not detected (lanes 1, 3, 7 and 9) or is detected in a manner in which there are significantly more nonspecific bands when compared to detection with Eco SSB (lanes 5 and 11 compared to lanes 6 and 12).

The effect of temperature on stringency of hybridization is well characterized. At lower temperatures, higher amounts of mismatched base pairs are tolerated in a hybridized duplex. The effect of lower stringency therefore is to increase the occurrence of duplex formation between single-stranded nucleic acid molecules that do not have homology or even substantial complementarity. That is, under conditions of low stringency a duplex can be formed between a primer/probe and a nucleic acid sequence that is not the target sequence desired, ie, a nonspecific hybridization product. Nonspecifically hybridized duplex DNA molecules can initiate primer extension and lead to the formation of nonspecific

amplified nucleic acid molecules.

The effects of a hybridization temperature that produces conditions of lower hybridization stringency on a PCR detection system containing Eco SSB is shown in Figure 3. As before at 54C, Eco SSB substantially improved the signal-to-noise ratio of detection of specific nucleic acids over non-specific nucleic acids when PCR was conducted using a 42C hybridization temperature. Whereas reducing the hybridization temperature lowered the stringency and therefore increase the nonspecific signal (compare lanes 9 to lane 3), Eco SSB substantially reduced the amount of detectable nonspecific duplex DNA under conditions of low hybridization stringency such as 42C (compare lane 3 to 4, or lane 5 to 6). Therefore the use of Eco SSB in a PCR detection system or method allows for less precision in determining the critical hybridization temperature and hybridization stringency required for conducting PCR or other hybridization based methods.

The results in Figure 3 also indicate that primers having shorter nucleotide lengths produce greater levels of nonspecific hybridization. This is not unexpected because primer length will effect the hybridization temperature (T_m). For example, compare lane 11 to lane 9, or compare lane 5 to lane 3, in which a 23 nucleotide primer exhibits more specificity than an 18 nucleotide primer. When using Eco SSB, the 18 nucleotide primer detects a specific sequence (lanes 3 and 10) not detectable in the absence of Eco SSB (lanes 2 and 9), at both 54C and 42C. Taken together, these data show that the use of Eco SSB improves specificity and reduces the need for precision in choosing and controlling hybridization stringency in a PCR detection method, whether the

determinant of stringency is hybridization temperature or amount of polynucleotide complementarity.

Additional studies were conducted on primers having regions of non-homology to the target sequence to determine the effect of Eco SSB on detection of the target sequence in a PCR detection method using primers having substantial complementarity. Hybridization reaction admixtures were prepared by the above-described methods and using Taq DNA polymerase, except that 100 ng of human genomic DNA was admixed with the primer pair pr 1A and pr 1B, or with the primer pair pr 2A and pr 2B, in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 250 ng of Eco SSB, and the prepared admixtures were subjected to the above PCR cycles with a 54C hybridization temperature. The results are shown in Figure 4.

The two primer pairs have sequences that were derived from the human dihydrofolate reductase gene (DHFR), at exon 1 (pr 1A and pr 1B), or at exon 2 (pr 2A and 2B). However, each primer has 18 nucleotide residues at their 5' ends that are not homologous to the DHFR gene, but rather correspond in sequence to portions of a universal lambda primer. At the 3' end of each primer there is a stretch of 33 nucleotides (pr 1A), 27 nucleotides (pr 1B), 27 nucleotides (pr 2A), or 30 nucleotides (pr 2B) that correspond to portions of the human DHFR gene. Thus, when specifically hybridized to the human genomic DNA DHFR gene, the duplex containing these primers has a 5' "tail" region comprising 18 nucleotides that is not complementary or hybridized to the template and is attached to the remaining 3' portion comprising 27 to 33 nucleotides that is hybridized to the DHFR gene.

The above DHFR primers are therefore examples of primers having substantial complementarity with the target sequence.

As shown in Figure 4, the two DHFR primer pairs pr 1A and pr 1B (lane 3) or pr 2A and pr 2B (lanes 1 and 2) detect a specific nucleic acid sequence by the PCR method that produces an amplified duplex DNA molecule of about 250 bp. Although the pr 2A and pr 2B primer pair yielded a specific band both in the presence or absence of Eco SSB (lanes 1 and 2), the pr 1A and pr 1B primer pair only yielded a specific band when Eco SSB was present (lane 3). Although the reason for this difference between the two primer pairs is not presently identified, an improvement in the PCR detection method was demonstrated by the data in Figure 4 for one of the two primer pairs tested by including Eco SSB in the PCR method. These results therefore demonstrate that primers with substantial but not complete complementarity are effective for use in combination with an SSB protein in the presently disclosed PCR detection method.

3. PCR Primer Studies

To examine the effects of E.coli SSB on PCR amplifications with perfectly complementary pairs of primers and templates, mixtures of each of 20 primer:template combinations were amplified with and without E. coli single stranded binding protein (SSB).

E.coli SSB was purified from the recombinant E.coli strain K12-H1 TRP/pTL119A-5 provided by Dr. Timothy Lohman. Purification of SSB was performed as described by Lohman, et al., Schwarz, et al., Nucl. Acids Res., 18:1079. One unit (1 ul) of

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SSB/PCR solution contained 500 ng SSB and 100 ng nuclease free bovine serum albumin (BSA) as carrier proteins in 50 mM Tris-Cl [tris (hydroxymethyl)amino methane HCl] pH 7.5, 500 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM 2-mercaptoethanol, 50 volume percent glycerol and 0.1 volume percent polyoxethylene lauryl ether (Lubrol-PX from Pierce Chemical). BSA was included in the SSB/PCR formulation because it inactivates PCR inhibitors, Tindall, K. R. and Kunkel, T. A., Biochemistry, 27:9253-9257, 1988. The nonionic detergent, lubrol, was also included because it assists in stabilizing Taq polymerase at elevated temperatures. Polymerase chain reactions were performed as described in the GeneAmp kit (Perkin-Elmer Cetus), in 100 ul volumes containing 2.5 units Taq DNA polymerase, 100-250 ng genomic template and 250 ng of each primer. PCR reactions with SSB contained 1 unit SSB/PCR solution.

Reactions were incubated at 95°C for 5 min to denature the genomic DNA template completely, then at 54°C for 5 min to allow primer annealing. SSB/PCR solution and Taq polymerase were added, and samples were amplified 30 cycles in a DNA thermal cycler using the following parameters: DNA denaturation for 1 min at 95°C, primer annealing for 1-3 min at 54°C, and primer extension for 1-2 min at 72°C. After the final cycle, samples were incubated at 72°C for 10 min.

For studies using matched primers, genomic DNA was prepared from human, rat, and mouse tissues and *E. coli* as described, Kechovono, P. and Thilly, W. G. Proc. Natl. Acad. Sci., 86:9253-9257, 1989; Paabo, S. Gifford, J. A. and Wilson, A. C. Nucl. Acids Res., 16:9775-9787, 1988. Gorilla and zebra DNA samples

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were-kindly provided Dr. by the San Diego Zoo. Messenger RNA, derived from rat and mouse tissues, was used to prepare cDNA as described Kechovono, P. and Thilly, W. G. Proc. Natl. Acad. Sci., 86:9253-9257, 1989. In the presence of SSB, 15 of 20 reactions show an increase in the yield of a primary product relative to secondary products, Jacobs, K. A., et al. Nucl. Acids Res., 16:4637-4650, 1988.

The effect of SSB was studied further by examining the patterns of products from PCR reactions using primers with mismatches at different locations.

The model system employed for the internal and 3' terminal primer mismatch studies used a transgenic mouse DNA template containing 1-2 copies per genome of a phage lambda L2B derivative, Nassal, M. and Rieger, A. Nucl. Acids Res., 18:3077, 1990. The mismatched primers were 23-mers complementary to the coding strand of the lacZ gene carried by phage lambda and combined internal single and double mismatches and deletions relative to the transgenic mouse DNA template. All possible terminal and penultimate mismatches were made for primers with the 3'ends: AA, GG, TT and CC. The downstream primer, used in all the mismatch experiments, was complementary to the non-coding strand at basepairs 19012-19034 of phage lambda Reiss, J. Klawczak, M., Schloesser, M., Wagner, M., Cooper, D. N., et al., Nucl. Acids Res. 18:973-978, 1990.

The template used for this set of experiments was genomic DNA from a transgenic mouse. The primers were constructed so as to contain internal mismatches. Mismatches at the 5' end of the primer, and most single internal mismatches and deletions, did not hamper the ability of SSB to increase primary product yield. Similar results were observed for

reactions using primers with double mismatches, unless a 3' terminal mismatch was involved.

Therefore, the effects that single basepair mismatches at or near the 3' end of a primer have on the spectrum of PCR amplification products in reactions with and without SSB were examined. PCR amplifications with primers having terminal G:A, A:G and C:C mismatches yielded almost no detectable primary product. With SSB, even lower yields of product are observed. All 3' terminal mismatches result in lower product yields in reactions containing SSB except: C:A, C:T and T:G.

The spectrum of products from amplification reactions with and without SSB using primers with 3' penultimate mismatches was examined. The resulting data show that the addition of E. coli SSB to reactions with these mismatched primers also result in reduced PCR product yields. The most dramatic decreases occur in amplification reactions with SSB when the penultimate 3' mismatch is: G:A or T:C.

All primers with 3' terminal double mismatches result in a substantial reduction of primary PCR product yields with and without SSB. However, in samples amplified without SSB, amplifications performed with several of these mismatched primers produced a broad molecular size distribution of secondary PCR products (amplification artifacts). In most cases, when SSB was used in these double mismatch amplifications, no PCR products could be detected.

The foregoing description and the examples illustrate the present invention but are not intended to limit the scope of the invention. Those skilled in the art recognize modifications and variations of the exemplified embodiments that are within the

spirit and scope of the invention described and
claimed herein.

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What-is claimed is:

1. A method for hybridizing a polynucleotide to a target nucleic acid, comprising the steps of:

5 a) forming a hybridization reaction mixture by admixing effective amounts of (i) a polynucleotide, (ii) a target nucleic acid, (iii) a divalent cation, and (iv) an isolated single-stranded nucleic acid binding protein, said polynucleotide probe having a sequence substantially complementary to a predetermined sequence present on the target nucleic acid; and

10 b) maintaining the hybridization reaction mixture under hybridizing conditions for a time period sufficient for the polynucleotide probe to hybridize to complementary nucleic acid sequences present in the target nucleic acid and form a hybridization product.

2. The method of claim 1 wherein said single-stranded nucleic acid binding protein is selected from the group consisting of Eco SSB, T4 gene 32 protein and T7 SSB.

3. The method of claim 1 wherein said divalent cation is selected from the group consisting of Mg^{++} , Mn^{++} , Ca^{++} and Zn^{++} .

25 4. The method of claim 1 wherein said divalent cation is Mg^{++} .

5. The method of claim 1 wherein said effective amount of divalent cation is in the range of 0.5 mM to 20 mM.

30 6. The method of claim 1 wherein said single-stranded nucleic acid binding protein is Eco SSB and said divalent cation is Mg^{++} present at a concentration of about 15 mM.

35 7. The method of claim 1 wherein said effective amount of single-stranded nucleic acid

binding protein is in the range of 1 ng to 10 ug per 100 ng of target nucleic acid.

5 8. The method of claim 1 wherein said nucleic acid is present in the hybridization reaction admixture affixed to a solid support.

 9. A method for detecting the presence of a specific nucleic acid sequence in a sample containing a nucleic acid, comprising the steps of:

10 a) forming a hybridization reaction mixture by admixing effective amounts of (i) a polynucleotide probe, (ii) a target nucleic acid, (iii) a divalent cation, and (iv) an isolated single-stranded nucleic acid binding protein, said polynucleotide probe having a sequence substantially
15 complementary to a predetermined sequence present on the target nucleic acid;

 b) maintaining the hybridization reaction mixture under hybridizing conditions for a time period sufficient for the polynucleotide probe
20 to hybridize to complementary nucleic acid sequences present in the target nucleic acid and form a hybridization product; and

 c) detecting the presence of any hybridization product formed in step (b) thereby
25 detecting the presence of the specific nucleic acid sequence in the nucleic acid.

 10. The method of claim 9 wherein said step (c) comprises detecting an amplified nucleic acid product and thereby the presence of a specific
30 nucleic acid sequence in said sample.

 11. The method of claim 10 wherein said amplified nucleic acid product is produced by a polymerase chain reaction.

 12. A method of preparing a primed template,
35 which method comprises:

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(a) forming a nucleic acid hybridization reaction admixture by admixing effective amounts of (i) a primer nucleic acid capable of specifically hybridizing to a template nucleic acid to form a substrate for polynucleotide synthesis, (ii) said
5 template nucleic acid, (iii) an isolated single-stranded nucleic acid binding protein, and (iv) a divalent cation; and

(b) maintaining said nucleic acid hybridization reaction admixture under hybridizing conditions for a time period sufficient for said
10 primer nucleic acid to specifically bind said template nucleic acid, thereby forming said primed template.

13. The method of claim 12 wherein said
15 single-stranded nucleic acid binding protein is selected nucleic acid binding protein is selected from the group consisting of Eco SSB, T4 gene 32 protein and T7 SSB.

14. The method of claim 12 wherein said
20 single-stranded binding protein is Eco SSB.

15. The method of claim 12 wherein said divalent cation is selected from the group consisting of Mg^{++} , Mn^{++} , Ca^{++} and Zn^{++} .

16. The method of claim 12 wherein said
25 divalent cation is Mg^{++} .

17. The method of claim 12 wherein said effective amount of divalent cation is in the range of 0.5 mM to 20 mM.

18. The method of claim 12 wherein said
30 single-stranded nucleic acid binding protein is Eco SSB and said divalent cation is Mg^{++} present at a concentration of about 15 mM.

19. The method of claim 12 wherein said
35 effective amount of single-stranded nucleic acid

binding protein is in the range of 1 ng to 10 ug per 100 ng of template nucleic acid.

20. A method of preparing a strand of nucleic acid having a nucleotide sequence substantially complementary to a template nucleic acid, which method comprises:

(a) forming a primer extension reaction admixture by admixing said template nucleic acid with effective amount of (i) a polynucleotide synthesis primer having a nucleotide sequence substantially complementary to a portion of the template nucleic acid, (ii) an isolated single-stranded nucleic acid binding protein, (iii) a divalent cation, and (iv) an enzyme capable of inducing polynucleotide synthesis in the presence of a primed template; and

(b) maintaining the primer extension reaction admixture under polynucleotide synthesizing conditions for a time period sufficient for said enzyme to produce a primer extension product, thereby producing said strand of nucleic acid.

21. The method of claim 20 wherein said single-stranded nucleic acid binding protein is Eco SSB.

22. The method of claim 20 wherein said divalent cation is selected from the group consisting of Mg^{++} , Mn^{++} , Ca^{++} and Zn^{++} .

23. The method of claim 20 wherein said effective amount of single-stranded nucleic acid binding protein is in the range of 1 ng to 10 ug per 100 ng of template nucleic acid, and said divalent cation is Mg^{++} present at a concentration of 1.5 mM.

24. A method of preparing a strand of DNA having a nucleotide sequence substantially complementary to a template nucleic acid, which method comprises:

(a) forming a primer extension reaction admixture by admixing said template nucleic acid with effective amounts of (i) a polynucleotide synthesis primer having a nucleotide sequence substantially complementary to a portion of the template nucleic acid, (ii) isolated Eco SSB, (iii) Mg^{++} , and (iv) a DNA polymerase; and

(b) maintaining the primer extension reaction admixture under polynucleotide synthesizing conditions for a time period sufficient for said DNA polymerase to produce a primer extension product, thereby producing said strand of DNA.

25. A method of amplifying a specific nucleic acid sequence present in a sample containing a double-stranded nucleic acid molecule, which method comprises:

(a) separating the strands of the double-stranded nucleic acid molecule to form single-stranded templates;

(b) treating the single-stranded templates with effective amounts of (i) primers that are selected so as to be substantially complementary to portions of the different strands of the specific double-stranded sequence to hybridize therewith such that an extension product synthesized from one primer, when it is separated from its template nucleic acid strand, can serve as a template for synthesis of the extension product of the other primer, (ii) an isolated single-stranded nucleic acid binding protein (SSB), (iii) a divalent cation, and (iv) an enzyme capable of producing primer extension products from said single-stranded templates, wherein said treatment is conducted under conditions such that the primers hybridize to the specific sequences and an extension product of each primer is

synthesized by the action of the enzyme, said product being substantially complementary to each single-stranded template;

5 (c) separating the primer extension products formed in step (b) from the templates on which they are synthesized to produce single-stranded molecules; and

10 (d) treating the single-stranded molecules produced in step (c) with effective amounts of the primers, SSB, divalent cation and enzyme of step (b) under conditions such that a primer extension product is synthesized using the single-stranded molecules formed in step (c) as a template.

15 26. The method of claim 25 wherein said single-stranded nucleic acid binding protein is selected from the group consisting of Eco SSB, T4 gene 32 protein and T7 SSB.

20 27. The method of claim 25 wherein said enzyme is a DNA polymerase selected from the group consisting of Klenow fragment, Taq DNA polymerase, recombinant Taq DNA polymerase, T7 DNA polymerase, modified T7 DNA polymerase and T4 DNA polymerase.

25 28. The method of claim 25 wherein said divalent cation is selected from the group consisting of Mg^{++} , Mn^{++} , Ca^{++} and Zn^{++} .

29. The method of claim 28 wherein said effective amount of divalent cation is in the range of 0.5 mM to 20 mM.

30 30. The method of claim 28 wherein said divalent cation is Mg^{++} present at a concentration of about 1.5 mM.

35 31. The method of claim 25 wherein said effective amount of single-stranded nucleic acid binding protein is in the range of 1 ng to 10 ug per 100 ng of template nucleic acid.

32. The method of claim 25 wherein said separating in steps (a) and (d) is accomplished by heat denaturing the double-stranded nucleic acid molecule.

5 33. The method of claim 32 wherein said single-stranded nucleic acid binding protein is heat stable.

34. The method claim 33 wherein said single-stranded nucleic acid binding protein is Eco SSB.

10 35. The method of claim 32 wherein said DNA polymerase is a heat-stable DNA polymerase.

36. The method of claim 35 wherein said heat-stable DNA polymerase is Taq DNA polymerase or recombinant Taq DNA polymerase.

15 37. A composition for use in a polymerase chain reaction to amplify a specific nucleic acid sequence comprising an isolated single-stranded nucleic acid binding protein and two polynucleotide primers that are selected so as to be sufficiently complementary to different strands of a duplex DNA containing said specific nucleic acid sequence to hybridize therewith such that an extension product synthesized from one primer, when it is separated from its complementary nucleic acid strand, can serve as a template for synthesis of the extension product of the other primer, and wherein said binding protein and primers are present in amounts effective to support a polymerase chain reaction.

20 38. The composition of claim 37 further including a DNA polymerase in an amount effective to support said polymerase chain reaction.

25 39. The composition of claim 38 wherein said DNA polymerase is a heat-stable DNA polymerase.

30 40. The composition of claim 37 wherein said single-stranded nucleic acid binding protein is

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selected from the group consisting of Eco SSB, T4 gene 32 protein and T7 SSB.

5 41. The composition of claim 37 wherein said single-stranded nucleic binding protein is heat-stable.

42. The composition of claim 41 wherein said heat-stable protein is Eco SSB.

10 43. The composition of claim 37 wherein said effective amount of single-stranded nucleic acid binding protein is in the range of 1 ng to 10 ug per 100 ng of template nucleic acid.

44. The composition of claim 37 further including an effective amount of a divalent cation.

15 45. The composition of claim 44 wherein said divalent cation is selected from the group consisting of Mg^{++} , Mn^{++} , Ca^{++} and Zn^{++} .

46. The composition of claim 44 wherein said effective amount of divalent cation is in the range of 0.5 mM to 20 mM.

20 47. The composition of claim 39 wherein said single-stranded nucleic acid binding protein is Eco SSB, and said composition further includes the divalent cation Mg^{++} in an amount sufficient to produce a concentration in said polymerase chain
25 reaction of 1.5 mM.

30 48. A composition for use in a primer extension reaction comprising an isolated single-stranded nucleic acid binding protein and a DNA polymerase, wherein said binding protein and DNA polymerase are present in amounts effective to support said primer extension reaction, said composition being free of primed template.

35 49. The composition of claim 48 wherein said single-stranded nucleic acid binding protein is selected from the group consisting of Eco SSB, T4

gene 32 protein and T7 SSB.

50. The composition of claim 48 wherein said effective amount of single-stranded nucleic acid binding protein is in the range of 1 ng to 10 ug per 100 ng of template nucleic acid.

51. A composition for enhancing polymerase chain reaction specificity, which composition comprises, in aqueous solution, single-stranded binding protein in an amount sufficient to enhance the production of primary polymerase chain reaction products and a non-ionic detergent in an amount sufficient to stabilize Taq polymerase against degradation during polymerase chain reaction amplification, said composition being free of nuclease activity.

52. The composition of claim 51 further including carrier protein at a concentration of 0.05 to 0.15 milligrams/milliliter.

53. The composition of claim 52 wherein said carrier protein is present at a concentration of 0.1 milligrams/milliliter.

54. The composition of claim 52 wherein said carrier protein is serum albumin.

55. The composition of claim 51 further including a chelating agent at a concentration sufficient to provide a cation-chelating capacity corresponding to that which 0.5 to 1.5 mM ethylenediaminetetraacetic acid would provide in said composition.

56. The composition of claim 55 wherein said chelating agent concentration is sufficient to provide a cation-chelating capacity corresponding to that which 1 mM ethylenediaminetetraacetic acid would provide in said composition.

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57. The composition of claim 51 further including 1 mM ethylenediaminetetraacetic acid.

58. The composition of claim 51 further including a buffering agent at a concentration sufficient to provide a buffering capacity corresponding to that which 0.05 to 500 mM tris(hydroxymethyl)aminomethane would provide in said composition.

59. The composition of claim 58 wherein said buffering agent concentration is sufficient to provide a buffering capacity corresponding to that which 50 mM tris(hydroxymethyl) aminomethane would provide in said composition.

60. The composition of claim 51 further containing 50 mM tris(hydroxymethyl)aminomethane.

61. The composition of claim 51 further including salt at a concentration sufficient to maintain the solubility of said single-stranded binding protein in said composition.

62. The composition of claim 61 further containing a salt at a concentration of at least 300 mM.

63. The composition of claim 61 further contain a salt concentration of 500 mM.

64. The composition of claim 61 wherein said salt is NaCl.

65. The composition of claim 51 further including an anti-freezing agent at a concentration sufficient to prevent freezing of said composition at -30 degrees centigrade.

66. The composition of claim 65 wherein said anti-freezing agent is glycerol or polyethylene glycol.

67. The composition of claim 51 further including 50 volume percent glycerol.

68. The composition of claim 51 further including a reducing agent at a concentration sufficient to provide a reducing capacity corresponding to that which 0.5 to 2 mM 2-mercaptoethanol would provide in said composition.

69. The composition of claim 68 wherein said reducing agent concentration is sufficient to provide a reducing capacity corresponding to that which 1 mM 2-mercaptoethanol would provide in said composition.

70. The composition of claim 51 further including 1 mM 2-mercaptoethanol.

71. The composition of claim 51 having a pH value of 7 to 8. /

72. The composition of claim 51 being free of nucleic acid.

73. The composition of claim 51 wherein said non-ionic detergent is polyoxyethylene lauryl ether.

74. The composition of claim 73 wherein said polyoxyethylene lauryl ether is present at a concentration of at least 0.1 volume percent.

75. The composition of claim 1 wherein said single-stranded nucleic acid binding protein is selected from the group consisting of Eco SSB, T4 gene 32 protein and T7 SSB.

76. The composition of claim 51 wherein said single-stranded binding protein is present at a concentration of at least 0.01 milligrams/milliliter.

77. A composition for enhancing polymerase chain reaction specificity, which composition comprises, in aqueous solution, single-stranded nucleic acid binding protein at a concentration of at least 0.1 milligrams/milliliter and non-ionic detergent at a concentration of at least 0.1 volume percent, said composition being free of nuclease activity.

78. A composition for enhancing polymerase chain reaction specificity, which composition comprises, in aqueous solution, 0.5 milligrams/milliliter single-stranded binding protein, 0.1 milligrams/milliliter carrier protein, 50 mM tris(hydroxymethyl)aminomethane; 500 mM NaCl; 1 mM ethylenediaminetetraacetic acid; 1 mM 2-mercaptoethanol; 50 volume percent glycerol, and 1 volume percent polyoxyethylene lauryl ether, said composition being free of nuclease activity and having a pH value in the range of 7 to 8.

79. The composition of claim 78 wherein said single-stranded nucleic acid binding protein is selected from the group consisting of Eco SSB, T4 gene 32 protein and T7 SSB.

1 / 2

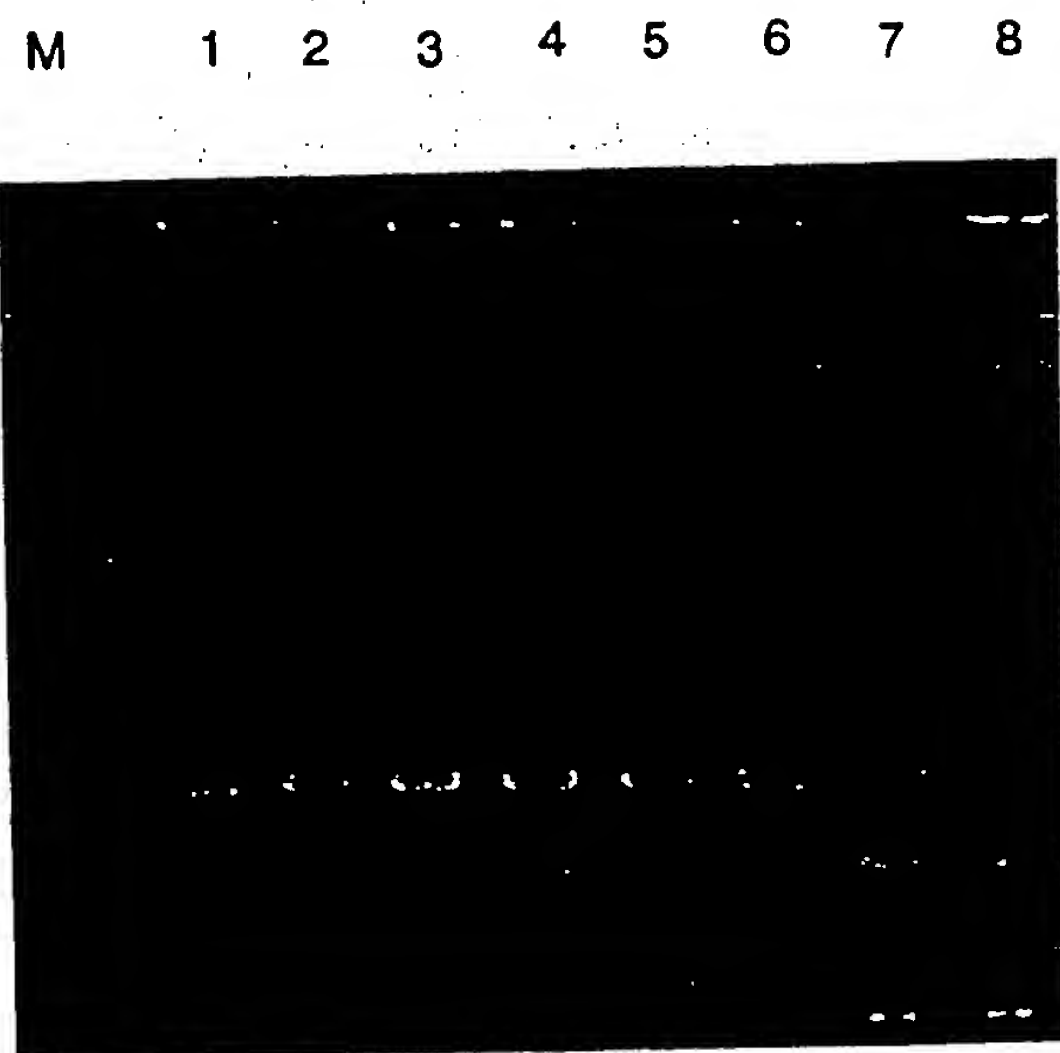


FIG. 1a

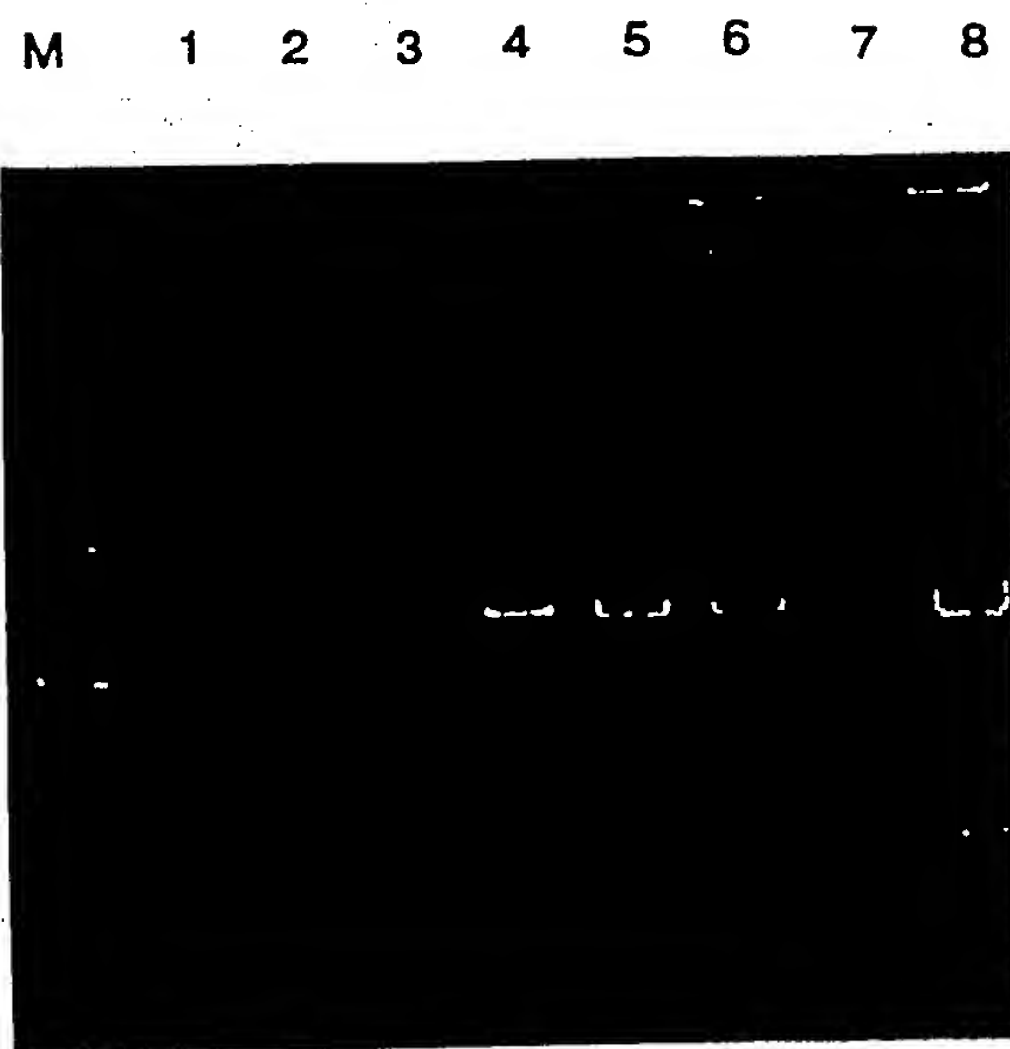


FIG. 1b

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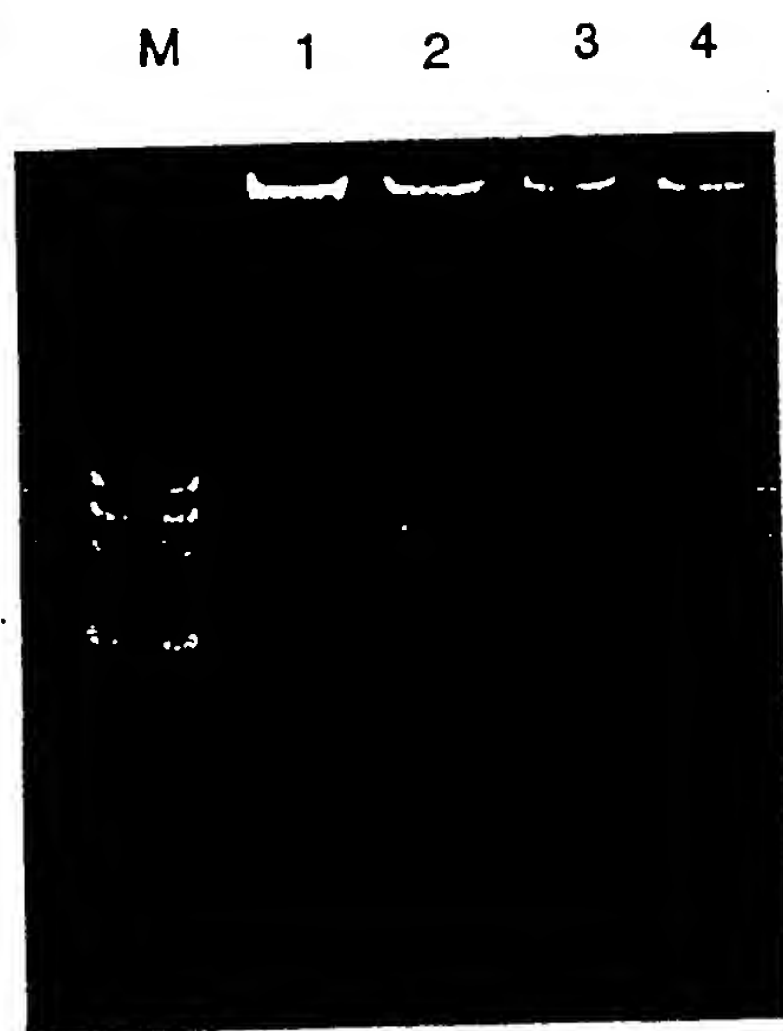


FIG. 4

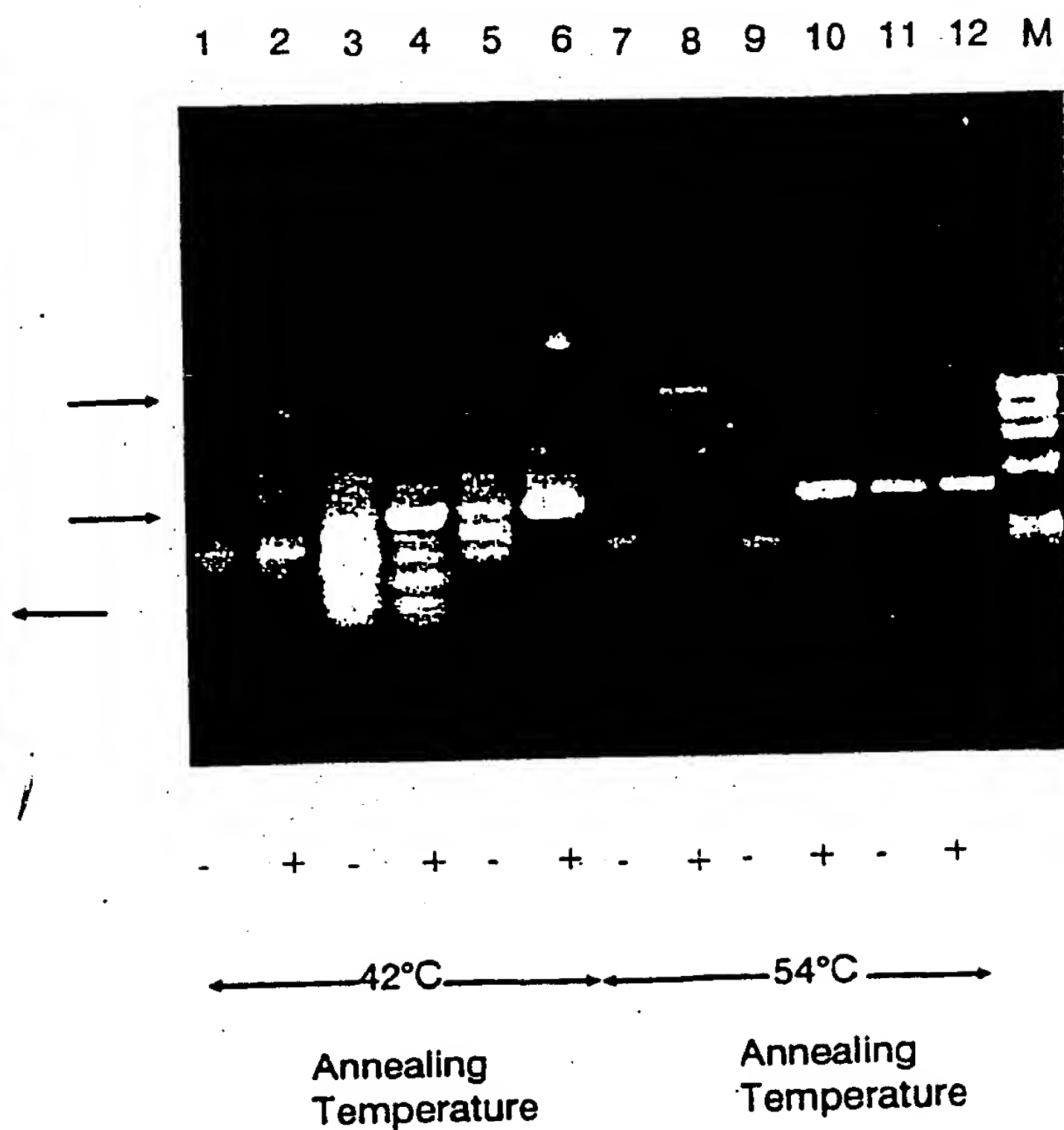


FIG. 3

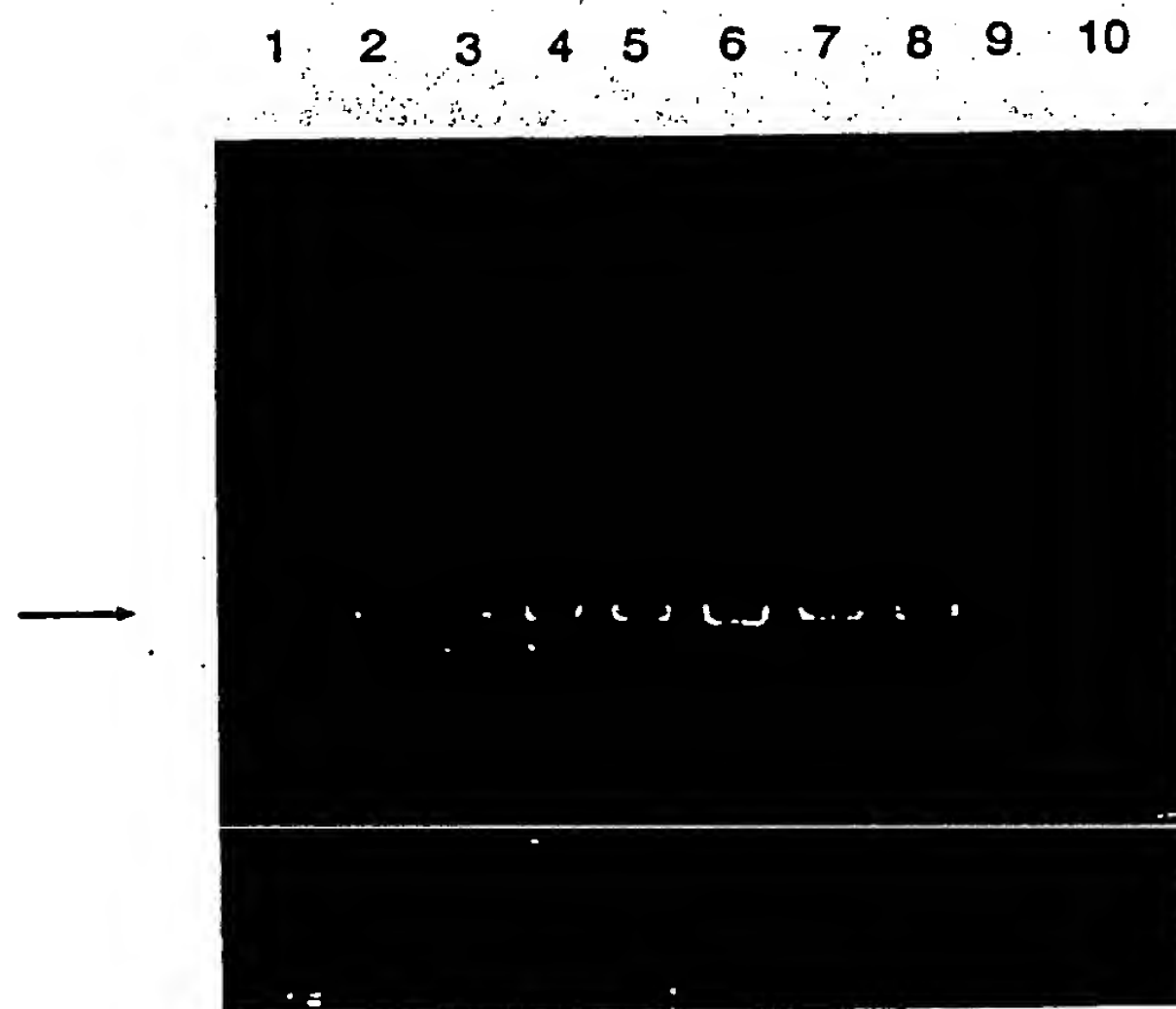



FIG. 2

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/06219

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ¹		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC(5):C12Q 1/68; C07H 15/12; C12N 15/00		
U.S. CL.: 435/6; 536/27; 935/77,78		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	435/6; 536/27; 935/77,78	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁵		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹¹		
Category ⁸	Citation of Document, ¹⁰ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	US, A. 4,688,195 (MULLIS et al.) 28 July 1987. see especially claim 1.	1-79
X	Journal of Molecular Biology, Volume 115. issued 1977, C. Christiansen et al., "Catalysis of DNA Reassociation by the <u>Escherichia</u> <u>coli</u> DNA Binding Protein", pages 441-454. see entire document.	1-79
<p>¹⁴ Special categories of cited documents: ¹⁵</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ¹⁶	Date of Mailing of this International Search Report ¹⁷	
02 January 1991	21 FEB 1991	
International Searching Authority ¹⁸	Signature of Authorized Officer ¹⁹	
ISA/US	 Mindy B. Fleisher	
		ebw